Effects of Various Chemical Constituents of Momordica charantia Fruits and Seeds and Other Plants on Lipid Metabolism in Isolated Rat Adipocytes

by

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To my father

We accept this thesis as confirming to the required standard for the degree of

MASTER OF PHILOSOPHY

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"Eureka!"

—— Archimedes

(287 - 212 B.C.)

ABSTRACT

The effects of a saponin, a lectin and two abortifacient proteins from Momordica charantia seeds on lipid metabolism in isolated rat adipocytes were studied. Compounds of similar chemical nature from Trichosanthes kirilowii (Tianhuafen), ginseng and other plants were also examined to see how their effects on lipid metabolism compared with those of compounds from M. charantia seed.

M. charantia saponin acted as a noncompetitive inhibitor of corticotropin, glucagon and epinephrine in lipolysis, and it also antagonized dibutyryl cyclic AMP-induced lipolysis. Incorporation of tritiated glucose into lipid was inhibited. Adipocyte viability was not affected by the saponin suggesting that its inhibitory effects on lipolysis and lipogenesis were not due to a decrease in cell viability.

Ginsenoside Rg_1, Rb and Rc noncompetitively inhibited corticotropin and dibutyryl cyclic AMP-induced lipolysis but the basal glucose incorporation into lipid was not affected.

All three abortifacient proteins, α-momorcharin, β-momorcharin and α-trichosanthin, possessed no intrinsic lipolytic, antilipolytic or lipogenic activity. However, incubation of corticotropin with either α- or β-momorcharin for several hours at room temperature resulted in an impairment of the lipolytic activity of the hormone.

M. charantia lectin competitively inhibited epinephrine- and glucagon-induced lipolysis but noncompetitively

inhibited corticotropin-induced lipolysis. Its inability to antagonize dibutyryl cyclic AMP-induced lipolysis suggests that the loci of action of the lectin in the adipocyte lie on the plasma membrane and that cell viability was not affected.

Tianhuafen lectin stimulated lipogenesis in rat adipocytes but did not inhibit epinephrine- or corticotropin-induced lipolysis in rat or hamster adipocytes. At a dose that did not stimulate lipolysis on its own, the lectin potentiated the lipolytic effects of corticotropin and epinephrine.

The effects of other plant lectins that specifically bind mannose, fucose, galactose, N-acetylgalactosamine and N-acetylglucosamine respectively, on lipolysis in isolated hamster and rat adipocytes and lipogenesis in rat adipocytes, were also examined. The relative potencies of the lectins in antagonizing epinephrine-induced lipolysis in rat adipocytes were M. charantia lectin (galactose binding) > Concanavalin A, garden pea lectin, lentil lectin (mannose binding) > wheat germ lectin (N-acetylglucosamine binding) and Wisteria floribunda lectin (N-acetylgalactosamine binding). The relative lipogenic potencies of the lectins were Concanavalin A, garden pea lectin, lentil lectin > wheat germ lectin > Maclura pomifera lectin (N-acetylgalactosamine binding), Wisteria floribunda lectin, and M. charantia lectin. The fucose binding gorse and asparagus pea lectins had minimal antilipolytic and lipogenic activities. The galactose binding horse gram and Bauhinia purpurea lectins had slight lipogenic activity and no antilipolytic activity in rat adipocytes but they exerted antilipolytic activity in hamster

adipocytes. Other galactose and N-acetylgalactosamine binding lectins tested including Cytisus sessilifolius, peanut, Bandeiraea simplicifolia II, soybean, Phaseolus limensis and Sophora japonica exhibited minimal antilipolytic and lipogenic activities.

M. charantia fruit "polypeptide-p" extracted according to the method of Khanna et al. (1981) stimulated lipogenesis in rat adipocytes and inhibited corticotropin-induced lipolysis in hamster adipocytes. A fraction purified from the seed "polypeptide-p" by affinity chromatography on fetuin-agarose chromatography also showed lipogenic activity.

By using a modified version of the mammalian insulin purification scheme, one basic protein and one acidic protein exhibiting potent lipogenic and antilipolytic activities were isolated. Similarly when the insect insulin purification protocol was employed, three chromatographically related fractions with antilipolytic and lipogenic activities could be isolated. These observations indicate that non-lectin insulinomimetic compounds that are extractable with acidic ethanol are present in the fruits and seeds of M. charantia.

ABBREVIATIONS

The following abbreviations are used in this Thesis :

ACTH	:	Corticotropin
ATP	:	Adenosine triphosphate
BSA	:	Bovine serum albumin
Con A	:	Concanavalin A
DbcAMP	:	N^6, O^2-Dibutyryl adenosine 3', 5'-cyclic monophosphate
Dimethyl POPOP	:	1,4-Bis-2.2 (4 methyl-5-phenoloxazolyl) benzene
FFA	:	Free fatty acid
KRB	:	Krebs-Ringer bicarbonate buffer
P-peptide	:	Polypeptide-p

CONTENTS

1. GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

Insulin used in the treatment of diabetes mellitus is usually obtained in a very low yield from animal pancreas, namely, approximately one pound of pure insulin from 10,000 pancreas. Sideeffects resulting from administration of insulin of non-human origin to patients, including hypoglycemia associated with hyperinsulinism, allergic utricaria, angioedema, anaphylactic reaction and lipoatropy are well known (Larner, 1980). Although sulfonylureas, a group of oral hypoglycemic agents of which tolbutamide is a prototype, have become popular for the management of maturity-onset diabetes, they are ineffective in treating juvenile-onset diabetes. Biguanides, another group of oral hypoglycemic agent, can be used in juvenile diabetes because their primary effect is exerted on the peripheral tissues and not on β-cells (Tepperman, 1975). However, the sideeffects of biguanides treatment are quite serious. Hence, phenformin has now been removed from the market in the United States because of its tendency to cause severe lactic acidosis. So the search for a nontoxic and effective hypoglycemic drug or insulin substitute has long been the goal of diabetologists. It deserves mention that synthesis of human insulin with the current recombinant DNA technology also holds great promise.

1.1 Scientific basis of some folkloric hypoglycemic plant

Since plants have been used as medications, it is not surprising that some of them are associated with hypoglycemic activity. Farnsworth and Segelman (1971) estimated that over 200

plants have been scientifically investigated because of their folkloric use as diabetic remedies by various ethnic and geographical groups. Among them are Myrtillin extract from blueberry leaves, root extract of the devil's club (Fatsia horrida), extract of rose periwinkle (Vinca rosaeae), extract of raw cabbage; cundeamor, made from Momordica charantia in Puerto Rico; Amellin from Scoperia dulcis and staghorn shrub (Rhus typhina) (Marquis, 1977). However, many of them are too toxic to be useful. For example, two extremely potent hypoglycemic agents, hypoglycins A and B, have been isolated from the unripe fruit of the akee tree Blighia sapida. Both of them are cyclopropanoid amino acids, the causatic agents of the Jamaican vomiting sickness. Hypoglycin A, which is almost twice as toxic as hypoglycin B, may lower the blood glucose concentration to 10 mg per 100 ml. The mode of action of both hypoglycins is quite different from that of insulin. The toxins and their metabolites specifically inhibit isovaleryl CoA dehydrogenase and thus block fatty acids oxidation. This depletes liver glycogen and subsequently induces hypoglycemia (Tanaka et al., 1972). The hypoglycemic activity of some folkloric anti-diabetic plants frequently cannot be demonstrated experimentally in crude plant extracts although compounds purified from the extract exhibit potent hypoglycemic activity. The leaves of the Madagascan periwinkle Catharanthus roseus (Vinca rosea) have been used by people in the Philippines, South Africa, Australia, England and Natal as folkloric diabetic remedies. Proprietary preparations e.g. Covinca (South Africa) and Vinculin (England), which are essentially infusions of leaves of C.roseus are still marketed in

some of these countries as oral insulin substitute. But results of clinical trials of these drugs have turned out to be negative (Farnsworth, 1961). In 1962, Svoboda and his co-workers isolated a large number of alkaloids from C. roseus. They found that the alkaloids catharanthine, hydrochloride, leurosine sulfate, lochnerine, tetrahydroalstonine, vindoline, and vindolinine dihydrochloride produced varying degrees of hypoglycemic activity in normal fasting rats. Vindolinine dihydrochloride and leurosine sulfate proved to be more active hypoglycemic agents than tolbutamide (Svoboda et al., 1962). Another plant Tecoma stans was found to be ineffective in alloxan-induced diabetic rodents in spite of its legendary use in Mexican folk medicine (Nash, et al., 1950). It was not until 1966 that it was determined that the alkaloids tecomine and tecostanine, isolated from the leaves of Tecoma stans, exhibited hypoglycemic activity in laboratory animals with intact pancreas (Hammonda et al., 1966).

A number of very common edible plants have been shown to elicit hypoglycemia in laboratory animals under a variety of conditions although the active principles involved are still unknown. These plants include the bean (immature pod), olive (leaves), potato, wheat, celery, blackberry (leaves), sugar beet, and banana (flowers and roots). Table 1-1 presents an additional list of plants with hypoglycemic activity in laboratory animals but not effective enough for practical use.

Traditional Chinese medicine has a long history of using herbs to treat diabetes mellitus. For instance, in the medical book "On Symptoms and Signs of Various Diseases" written in the

Table 1-1

Some common plants showing experimental hypoglycemic activity

(Compiled after Farnsworth et al., 1971)

PLANT FAMILY	SCIENTIFIC NAME	COMMON NAME
Anacardiaceae	*Anacardium occidentale*	Cashew
Anacardiaceae	*Rhus typhina*	Staghorn sumac
Apocynaceae	*Rauwolfia serpentina*	Snakeroot
Araliaceae	*Panax ginseng*	Ginseng
Berberidaceae	*Hydrastis canadensis*	Goldenseal
Cactaceae	*Lophophora williamsii*	Peyote
Caricaceae	*Carica papaya*	Papaya
Chenopodiaceae	*Spinacia oleracea*	Spinach
Compositae	*Arctium lappa*	Burdock
Compositae	*Artemisia vulgaris*	Mugwort
Compositae	*Erigeron canadensis*	Horseweed
Compositae	*Eupatorium purpureum*	Joe-pye weed
Compositae	*Helianthus annuus*	Sunflower
Compositae	*Helianthus tuberosus*	Jerusalem artichoke
Compositae	*Lactuca sativa*	Lettuce
Compositae	*Taraxacum officinale*	Dandelion
Convolvulaceae	*Ipomoea batatas*	Sweet potato
Cruciferae	*Brassica oleracea*	Cabbage
Cruciferae	*Brassica rapa*	Turnip
Ericaceae	*Chimaphila umbellata*	Pipsissewa
Euphorbiaceae	*Euphorbia pilulifera*	Pill-bearing spurge
Geraniaceae	*Geranium maculatum*	Wild crane's-bill
Gramineae	*Avena sativa*	Oats
Gramineae	*Hordeum vulgare*	Barley
Gramineae	*Zea mays*	Corn
Labiatae	*Lycopus virginicus*	Bugleweed
Leguminosae	*Cassia occidentalis*	Senna
Leguminosae	*Lupinus albus*	Lupine
Leguminosae	*Phaseolus mungo*	Mung bean
Leguminosae	*Trigonella foenumgraecum*	Fenugreek
Liliaceae	*Allium cepa*	Onion
Liliaceae	*Allium sativum*	Garlic
Liliaceae	*Convallaria majalis*	Lily of the valley
Liliaceae	*Polygonatum officinale*	Solomon's-seal
Loganiaceae	*Gelsemium sempervirens*	Yellow jasmine
Lycopodiaceae	*Lycopodium clavatum*	Creeping Jennie
Lythraceae	*Lagerstroemia speciosa*	Queen crape myrtle
Musaceae	*Musa sapientum*	Banana
Myrtaceae	*Pimenta officinalis*	Allspice
Oleaceae	*Olea europaea*	Olive
Palmae	*Cocos nucifera*	Coconut
Papaveraceae	*Chelidonium majus*	Great celandine
Ranunculaceae	*Cimicifuga racemosa*	Black cohosh
Solanaceae	*Capsicum frutescens*	Red pepper
Solanaceae	*Nicotiana tabacum*	Tobacco
Taxaceae	*Taxus cuspidata*	Spreading yew
Umbelliferae	*Apium graveolens*	Celery
Umbelliferae	*Coriandrum sativum*	Coriander
Zingiberaceae	*Zingiber officinale*	Ginger

seventh century (Tang Dynasty), Lonicera japonica was advocated for treating the disease. In another famous medical book "One Thousands Golden Prescriptions", Trichosanthes kirilowii root and Coptis chinenesis root were reported to be popular remedies. Chen (1981) listed 17 plants that have been in common use since the ancient times either as single herbal medicine or as compound prescriptions for curing diabetes mellitus (Table 1-2).

In recent years, extensive research has been done on the hypoglycemic effect of the unripe fruit of M. charantia in laboratory animals and in humans. To date four insulin-like polypeptides of different molecular weight have been reportedly isolated from the fruit by investigators in Mainland China and in India (Zhang, et al.,1980; Khanna, et al., 1981). An insulin-like peptide was also purified from ginseng radix which has also been used as a folkloric diabetic remedy (Ando et al., 1980). However the observations of insulin-like peptides are not restricted to these two plant species. Mannose binding lectins comprising Con A, garden pea lectin and lentil lectin, N-acetylglucosamine binding wheat germ lectin, and galactose and N-acetylgalactosamine binding ricin I and II, can all bind to insulin receptors and evoke in vitro insulin-like effects (Katzen et al., 1981).

1.2 Aims and strategy of study

In the present study, an attempt was made to search for insulin-like or hypoglycemic components in the seeds of M. charantia. The hormone-induced lipolysis and lipogenesis assays were used to monitor insulin-like bioactivity during the course of isolation. Two purification schemes were adopted. One was originally used to purify insulin-like compound from insects and

Table 1-2

Some common herbal plants used in Chinese medicine to treat diabetes mellitus (compiled after Chen, 1981)

PLANT FAMILY	SCIENTIFIC NAME	PART USED
Anacardiaceae	*Rhus chinensis*	Gall
Araliaceae	*Panax ginseng*	Root
Caprifoliaceae	*Lonicera japonica*	Flower-bud
Cucurbitaceae	*Lagenaria siceraria*	Fruit
Cucurbitaceae	*Momordica charantia*	Fruit
Cucurbitaceae	*Trichosanthes kirilowii*	Root
Leguminosae	*Astragalus membranaceus*	Root
Liliaceae	*Asparagus cochinchinensis*	Root
Liliaceae	*Ophiopogon japonicus*	Root
Moraceae	*Morus alba*	Fruit
Moraceae	*Morus alba*	Inner-bark
Myrtaceae	*Psidium guajava*	Leaf
Ranunculaceae	*Coptis chinensis*	Root
Scrophulariaceae	*Rehmannia glutinosa*	Root
Scrophulariaceae	*Scrophularia buergerana*	Root
Solanaceae	*Lycium chinense*	Root-bark
Solanaceae	*Lycium chinense*	Fruit

annelids (LeRoith et al., 1981) while the other was a modification of the method developed by Mirsky (1973) to purify insulin from bovine pancreas. Theoretically, the most reasonable bioassay for monitoring the course of purification would be a measurement of the hypoglycemic activity of the fractions in diabetic animals. However, this method demands the use of a large number of experimental animals and a lot of space for holding the animals. It was reported that (Chen, 1981) the fruit extract of M. charantia, unlike tolbutamide, could not promote insulin secretion from the pancreas but resembled insulin in that it enhanced carbohydrate utilization. Because it is well established that insulin stimulates lipogenesis and inhibits hormone-induced lipolysis in fat cells, in vitro lipogenesis and hormone stimulated lipolysis assays using isolated fat cells were employed to monitor the isolation of insulin-like compounds from M. charantia seeds. The most active fraction(s) isolated were then tested for a possible hypoglycemic effect in animals with hyperglycemia. Although the emphasis of the work was placed on compounds from Momordica charantia, compounds of similar chemical natures from Trichosanthes kirilowii, ginseng and other plants were also examined to see how their effects on lipid metabolism compared with those of the M. charantia compounds.

1.3 Triacylglycerol metabolism in adipocytes

Adipose tissue stores energy in the form of fat : it converts excess glucose to triglycerides in preference over glycogen. It was first shown to be sensitive to insulin in 1951

(Krahl, 1951). It is now known that the tissue is also responsive to a large number of other hormones including epinephrine, norepinephrine corticotropin, glucagon, growth hormone, thyroxine, glucocorticoids, melanotropin, thyroid stimulating hormone, prolactin, vasopressin and luteinizing hormone (Ganong, 1981). Extensive research on the mechanism of hormones on glucose and lipid metabolism in adipose tissue strips has been done by using the release of fatty acids and glycerol as the end points.

However, this method suffers from the drawback that adipose tissue contains not only adipocytes but also stromal cells, blood vessel and mast cells etc. Variation in response among tissues and the problem of diffusion can be eliminated if a homogeneous population of adipocytes is used instead of adipose tissue strips.

1.3.1 Morphology of isolated adipocytes

Using crude bacterial collagenase to digest the connective tissue of epididymal fat pads, Rodbell (1964) successfully isolated a homogeneous population of adipocytes from rats weighing 160-210 g. Under the light microscope, each adipose cell consists of a large spherical lipid droplet surrounded by a thin rim of cytoplasm. It has a diameter of 50-100 microns, a cell surface area of 8000 u^2 (Lang *et al.*, 1974), and a mean dry weight of 130 ng.

Cell size increases linearly until the rat weighs about 600 g, but cell number stops to increase when the rat grows to a weight of 300-350 g (Hartman *et al.*, 1971). The mature white

adipocyte consists of roughly 95% triglyceride by weight. Ultrastructural studies (Cushman, 1970) reveal that, in addition to typical subcellular organelles, a variety of small lipid droplets and an extensive system of membranes characterize the cytoplasm. A fenestrated envelope surrounds the large, central lipid droplet. Similar envelopes surround cytoplasmic lipid droplets occuring individually or as aggregates of very small, amorphous droplets. Groups of individual droplets of small size also occur without envelopes. The system of membranes consists of invaginations of the cell membrane, vesicles possibly of pinocytic origin, simple and vesiculated vacuoles, vesicles deeper in the cytoplasm, flattened and vesicular smooth-surfaced endoplasmic reticulum, and Golgi complex. These observations suggest that the adipocyte has compartmentalized transport and metabolism.

Free fat cells maintain the different metabolic characteristics observed in adipose tissue (Rodbell, 1964). Like adipose tissue, they also exhibit the readily monitored cell-specific hormonal response, namely lipolysis which results in the release of free fatty acid and glycerol from the cell. Isolated adipocytes are very useful for studies on the effect of hormones and drugs on metabolism of lipid and carbohydrates because they do not have the problems of variations in response among different adipose tissue strips and inaccessibility of inner cells in the strips to added drugs.

1.3.2 Triglyceride accumulation in adipocytes (Lipogenesis)

The glyceryl moiety of triglyceride is derived from glucose transported into the adipocyte under the stimulation of insulin. Some fatty acids which incorporated into triglycerides are synthesized in the adipocyte from glucose. The remainder is delivered through the blood in the form of triglycerides contained in either chylomicrons or very low density lipoprotein. In both cases, insulin facilitates the hydrolysis of lipoprotein triglycerides by stimulating the production of lipoprotein lipase. On the other hand, insulin inhibits the hormone-sensitive lipase concerned with the mobilization of fatty acids. Fig. 1-1 shows the major metabolic effects of insulin in the adipocyte.

1.3.3 Fatty acid mobilization from adipocytes (Lipolysis)

To leave the adipocyte, triglycerides must be hydrolyzed to fatty acids and glycerol. During lipolysis, both free fatty acids (FFA) and glycerol are released from the cell. Glycerol produced by lipolysis cannot be used by adipocytes because they lack the enzyme glycerol kinase. Some of the FFA are reesterified in the cell (Patten, 1970).

The general aspects of adipocyte function and lipid release are summarized in Fig. 1-2. Norepinephrine, epinephrine, corticotropin and glucagon activate the adipocyte triglyceride lipase, also called the hormone-sensitive lipase. The first event in the sequence of reactions is the combination of these hormones with receptor sites on the cell membrane and consequent activation of adenylate cyclase, a membrane-bound enzyme that converts ATP to 3',5'-cyclic adenosine monophosphate (cAMP)

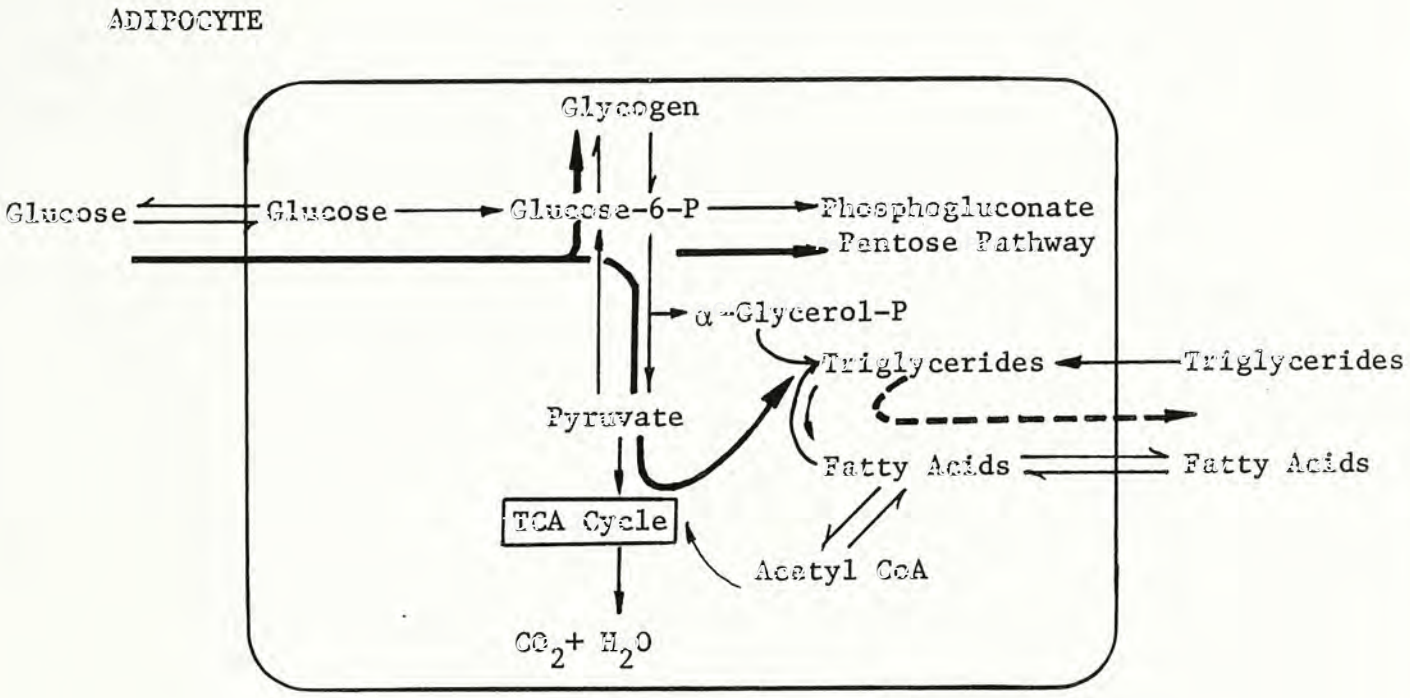


Figure 1-1. Major metabolic effects of insulin deficiency. Thick solid arrows (⟶) show the pathways that are favored in the presence of insulin, while thick broken arrows (⇢) depict those that predominate when the action of the hormone is insufficient. (Larner, 1980)

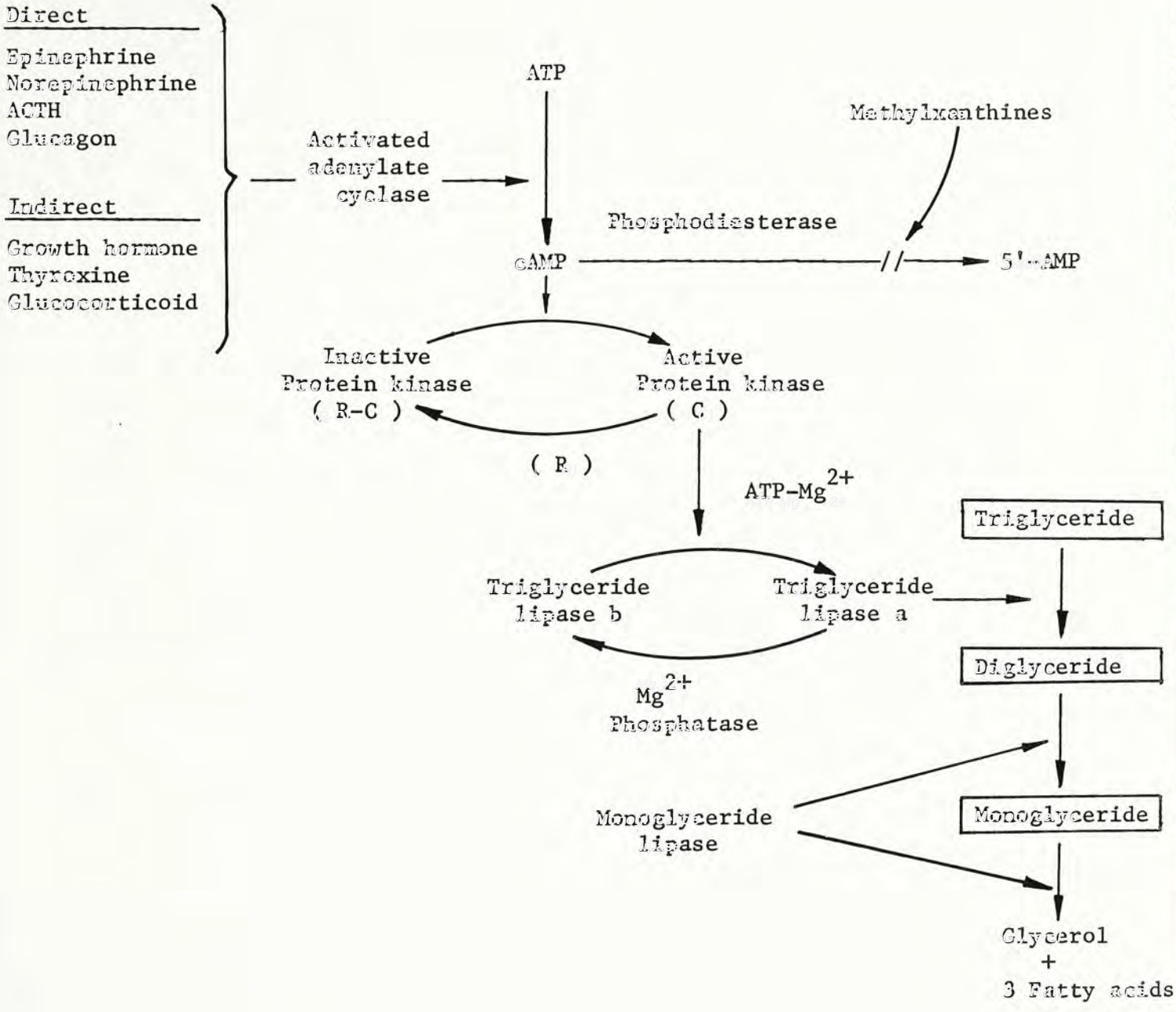


Figure 1-2. Fatty acid mobilization from adipocytes.

(Birnbaumer and Rodbell, 1969). Cyclic AMP interacts with the regulatory (R) subunit of protein kinase (Soderling et al., 1973). The catalytic (C) subunits released from the C:R protein kinase complex in turn catalyzes an ATP-induced phosphorylation of the hormone-sensitive lipase and transforms it in the presence of Mg^{2+} (Khoo et al., 1973) from an inactive triglyceride lipase (lipase b) into an active form (lipase a). The deactivation of the active lipase a is catalyzed by a protein phosphatase . The hydrolysis catalyzed by the hormone-sensitive lipase is the rate limiting step in triglyceride catabolism . Hydrolysis of the resulting diglyceride and subsequently, the monoglyceride to FFA and glycerol, is mediated by a single enzyme monoglyceride lipase (Severson et al., 1977).

Thyroxine and growth hormone act more slowly in stimulating lipolysis. They probably operate by increasing the synthesis of a regulatory protein rather than by activating existing adenylate cyclase (Ganong, 1981). Glucocorticoids also stimulate lipolysis, but they do so by facilitating the action of other fat-mobilizing hormones rather than by exerting a direct effect.

High levels of glucose and insulin can stimulate triglyceride accumulation in adipocytes. When this occurs, the cAMP content of the adipocyte is reduced. Methylxanthines such as caffeine and theophylline enhance fatty acid mobilization from adipocytes by inhibiting phosphodiesterase which inactivates cAMP. As a result, the increase in cAMP level maintains the hormone-sensitive lipase in active form.

1.4 Aged rats used as diabetic animal model

There are four commonly used experimental animal models with hyperglycemia : alloxan or streptozotocin-induced hyperglycemia, epinephrine-induced transient hyperglycemia and genetically diabetic animals. Alloxan and streptozotocin are specific in destroying pancreatic β-cells and thereby rendering the animals diabetic, but the sideeffects are so great that a large number of animals are required to allow for the great death rate (Boquist, 1980; Ganda et al., 1976). Also compounds producing hypoglycemia by increasing insulin secretion from β-cells would not be active in this assay system. Epinephrine-induced hyperglycemia is too transient so that substances with a slow effect may be undetectable by this method. Genetically diabetic rodents such as PBB/Ld mouse and db/db mouse could be used but there stands the possibility of interspecific variation in response to the test fractions (Hunt, 1976) because epididymal fat cells from Sprague-Dawley rats were used to monitor in vitro insulin-like bioactivity in the various fractions. Wexler (1981) reported that old Sprague-Dawley rats showed a tendency towards hyperglycemia, hyperlipidemia, increased blood urea nitrogen and other metabolic alternations found in maturity-onset diabetes. Hence they constitute a good model for testing the in vivo hypoglycemic effect of insulin-like compound(s) isolated from M. charantia seeds.

2. VARIOUS CHEMICAL CONSTITUENTS OF MOMORDICA CHARANTIA FRUITS AND SEEDS

2 VARIOUS CHEMICAL CONSTITUENTS OF MOMORDICA CHARANTIA FRUITS AND SEEDS

2.1 Introduction

M. charantia Linn of family Cucurbitaceae is widely cultivated in nearly all tropical and subtropical regions. Other vernacular names are bitter gourd, bitter melon (China), African cucumber, karela, carilla (India and Pakistan), ampalaye (Philippines) maiden apple, maiden's blush (West Indies), Cundeamor and balsamina (Cuba, Puerto Rico and Spanish-speaking tropical America) (Morton, 1977). It is an edible, medicinal and toxic plant. The unripe fruit is eaten in various ways, but the ripe friut is too toxic and bitter. The vines, fruits, seeds, leaves and roots in fresh and dry form are used in folk medicine for heat-stroke, dysentry, thrist from febrile symptoms, skin ulcers, detoxication (Anon, 1979) and sometimes as an emetic purgative and abortifacient (Morton, 1967). In some areas, especially Puerto Rico and Cuba, the fruit has long been used as folkloric diabetic remedies (Rivera, 1941).

2.1.1 Hypoglycemic activity of the unripe fruit

Hypoglycemic activity of orally administered juice was reported in normal and alloxan-induced diabetic rabbits (Sharma et al., 1960). Dried and powdered fruit also exhibited hypoglycemic activity (Akhtar et al., 1981). Lotlikar and Rao (1961) isolated "Charantin" from the fruit which Sucrow (1960) showed to consist of equal parts of β-sitosterol glucoside and 5,25-stigmastadiene-

ol glucoside. Charantin had a more potent hypoglycemic activity in normal fasting rats than tolbutamide (Sucrow, 1965). (Lotlikar and Rao, 1961) But it lacked any effect on alloxan-treated rats (Marquis et al., 1977). Results of clinical trials of a drug prepared from dried and powdered fruit in 29 maturity-onset diabetic patients were reported by a group of clinicians in Mainland China (Anon, 1977). These investigators demonstrated that the main symptoms of diabetes i.e. hyperglycemia, polyphagia as well as polydipsia were significantly alleviated. A crude extract of the fruit injected intraperitoneously also exerted a hypoglycemic effect in alloxan-induced diabetic mice (Anon, 1977). The fruit extract could not stimulate the secretion of insulin from β-cells, but could increase peripheral utilization of carbohydrate (Chen, 1981).

2.1.2 Insulin-like peptides

By using procedures involving acidic ethanol extraction, acetone precipitation, membrane dialysis, and crystallization with zinc chloride, Khanna et al. (1974) claimed to have isolated from the fruit, seeds and tissue of M. charantia, a hypoglycemic peptide which they termed "polypeptide-p"(p-peptide), with a minimum molecular weight of approximately 11,000 (166 residues). The polypeptide did not cross react in a radioimmunoassay for bovine insulin. However, Zhang et al (1980), using acidic ethanol extraction, ethanolic ether precipitation, gel filtration and ion-exchange chromatography, successfully isolated three groups of insulin-like peptides of molecular weight 8,000-

6,000, 2,500-2,000, and 1,200-800 respectively. All three groups possessed hypoglycemic effect in diabetic and normal fasting mice. They also inhibited the binding of ^{125}I-insulin to its antibodies and its hepatic receptors.

2.1.3 Abortifacient proteins

In the West India and tropical areas of Mexico, Central and South America, the fruit is a popular abortifacient (Morton, 1967). The fruit extract was reported to induce infertility in male dog (Dixit et al., 1978). Recently, two abortifacient proteins, α-momorcharin and β-momorcharin were purified from seeds in our laboratory. Both proteins were shown to be effective in inducing mid-term abortion in mice (Law, 1983; Chan, 1984).

2.1.4 Lectins

Lin et al. (1978) purified two galactose binding lectins from M. charantia seeds. One of them called M. charantia agglutinin, was a potent haemagglutinin, whereas the other one, called momordin, had lower hemagglutinating activity and inhibited protein synthesis in Ehrlich ascite tumor cells to a moderate extent. Both lectins were single polypeptide chains with molecular weight of 31,000 and 23,000 respectively. Horejsi and his co-workers (1980) purified a D-galactose binding lectin with a molecular weight of 12,900. Another D-galactose binding lectin of molecular weight 124,000 was isolated in our laboratory. Comparison of the chemical characteristics of our lectin preparation with those of the other groups revealed that our

preparation was chemically more similar to those of Horejsi *et al*'s and Barbieri *et al*'s preparations obtained by affinity chromatography, than to those of Li's and Lin *et al*'s preparations obtained by ion exchange chromatography (Barbieri *et al*., 1980; Li, 1980). Our lectin had a molecular weight of 124,000 which is very close to the values of 129,000 and 115,000 reported by Horejsi's group (Horejsi *et al*., 1980) and Barbieri's group (Barbieri *et al*., 1980) respectively. The molecular weights of the three lectin subunits reported by Horejsi's group are also similar to what we found : they all had a value of about 35,000. Barbieri's group, however, found that their lectin preparation consisted of four subunits each with a molecular weight of about 28,000.

2.1.5 Other bioactive factors

The ripe fruit and leaves contained a guanylate cyclase inhibitor which had the ability to impair chemical carcinogen-induced increase in guanylate cyclase activity (Vesely *et al*., 1977). The guanylate cyclase inhibitor was later found to be responsible for the cytotoxic effect of a crude fruit extract in human lymphocytes (Takemoto *et al*., 1980). A saponin was purified from seeds in our laboratory.

To date, much effort has been concentrated on the study of hypoglycemic activity of *M. charantia* fruits and little work has been done on the effects of the chemical constituents of *M. charantia* seeds on lipid metabolism *in vitro* and *in vivo*. Some

ginseng saponins have been found to inhibit corticotropin-induced lipolysis as well as insulin-induced lipogenesis in isolated rat adipocytes (Ohminami et al., 1981). It was therefore of interest to see whether the saponin isolated from seeds in our laboratory also exhibited similar biological activities. The insulinomimetic activity of some lectins e.g. Con A and wheat germ agglutinin are well established. The galactose binding lectin isolated in our laboratory was tested to see how the M. charantia lectin compared with other lectins in its insulin-like activity. To our knowledge, no reports have appeared on the examination of the "p-peptide" isolated from the fruits and seeds by Khanna et al. (1981) for in vitro insulin-like activities such as inhibition of lipolysis and stimulation of lipogenesis. Hence, we strictly followed the published procedure of Khanna and Jain (1981) and used it to isolated from the fruits and seeds of M. charantia "p-peptide" which we then tested for antilipolytic and lipogenic activities. It was not known whether any hypoglycemic insulin-like peptide(s), besides the four found in the fruits, existed in M. charantia seeds. An attempt was therefore made to search for this kind of peptide in the light of the finding that hypoglycemic "p-peptide" was also reported to be present in the seeds. Both momorcharins have deleterious effect on the implanting embryo as well as the endometrium (Chan et al., 1984). However, no studies on their metabolic effects especially those on glucose and lipid metabolism had so far been attempted. It is well-established that pregnant women tend to become diabetic because of the great glucose demand from the foetus (Zilva et al., 1979). Some placental hormones like human chorionic

somatomammotropin (human placental lactogen) secreted by syncytiotroplasts possess lipoytic effect (Ganong, 1981). In view of the dramatic endocrine and consequent metabolic changes in the female mammal associated with pregnancy, we decided to examine the possibility of a metabolic action of these proteins. The isolated rat epididymal adipocyte was selected as the *in vitro* model of study because, as mentioned before, it is well proven to be sensitive to hormones that regulate carbohydrate, protein and lipid metabolism such as insulin, glucagon, epinephrine and corticotropin (DiGirolamo *et al.*, 1976; Rodbell, 1964; Rudman, *et al.*, 1969; Zinman *et al.*, 1974). Adipocytes isolated from the male rat were used but it deserves mention that cells isolated from parametrial adipose tissue in the female rat have been demonstrated to be responsive to hormonal stimulus (Fain *et al.*, 1965).

2.2 Materials

Fresh unripe fruits of *M. charantia* Linn were obtained from local market. Dry and ripe seeds were purchased from local herbal medicinal stores. The suppliers of the chemicals used were listed in table 2-1. All other chemicals were of analytical grade or of the best quality available.

2.3 Methods

2.3.1 Fractionation and Purific[illegible]

All fractionation and puri[illegible]ati[illegible] were[illegible] ndertaken by

Table 2-1

Chemicals used and suppliers

CHEMICALS	SUPPLIERS
ACTH	Sigma,(St. Louis, Missouri)
L(+)-Ascorbic acid	Merck
BSA (Fraction V)	Sigma
α-Chymotrypsin	Worthington
Collagenase Type II	Sigma
Dbc AMP	Sigma
O-Dianisidine dihydrochloride	Sigma
4,5-Dihydroxynaphthalene-2,7-disulfonic acid disodium salt	Sigma
Dimethyl POPOP	Sigma
Emulsin (EC No. 3.2.1.2.1)	Sigma
Epinephrine bitartrate	Sigma
Galactose	Sigma
Glucagon	Sigma
D-Glucose	BDH
D-[3-3H] Glucose	Amersham
Glucose oxidase (PGO enzyme)	Sigma
Glutathione	Sigma
Heparin	Sigma
Insulin	Eli Lilly
Lima bean trypsin inhibitor	Sigma
α-Methyl-D-mannoside	Sigma
Olive oil	Sigma
Pronase E	Sigma
Propranolol	Imperial Chem.
Sodium pentobarbital	Serva
Theophylline	Sigma
Trypsin	Worthington
Vanillin	Sigma

Miss W.W. Li and Mr. K.C. Pong in our laboratory thoroughout the whole study. Details of the purification procedures and chemical characterization of the lectin, saponin, and abortifacient proteins will be published elsewhere.

2.3.1a M. charantia lectin

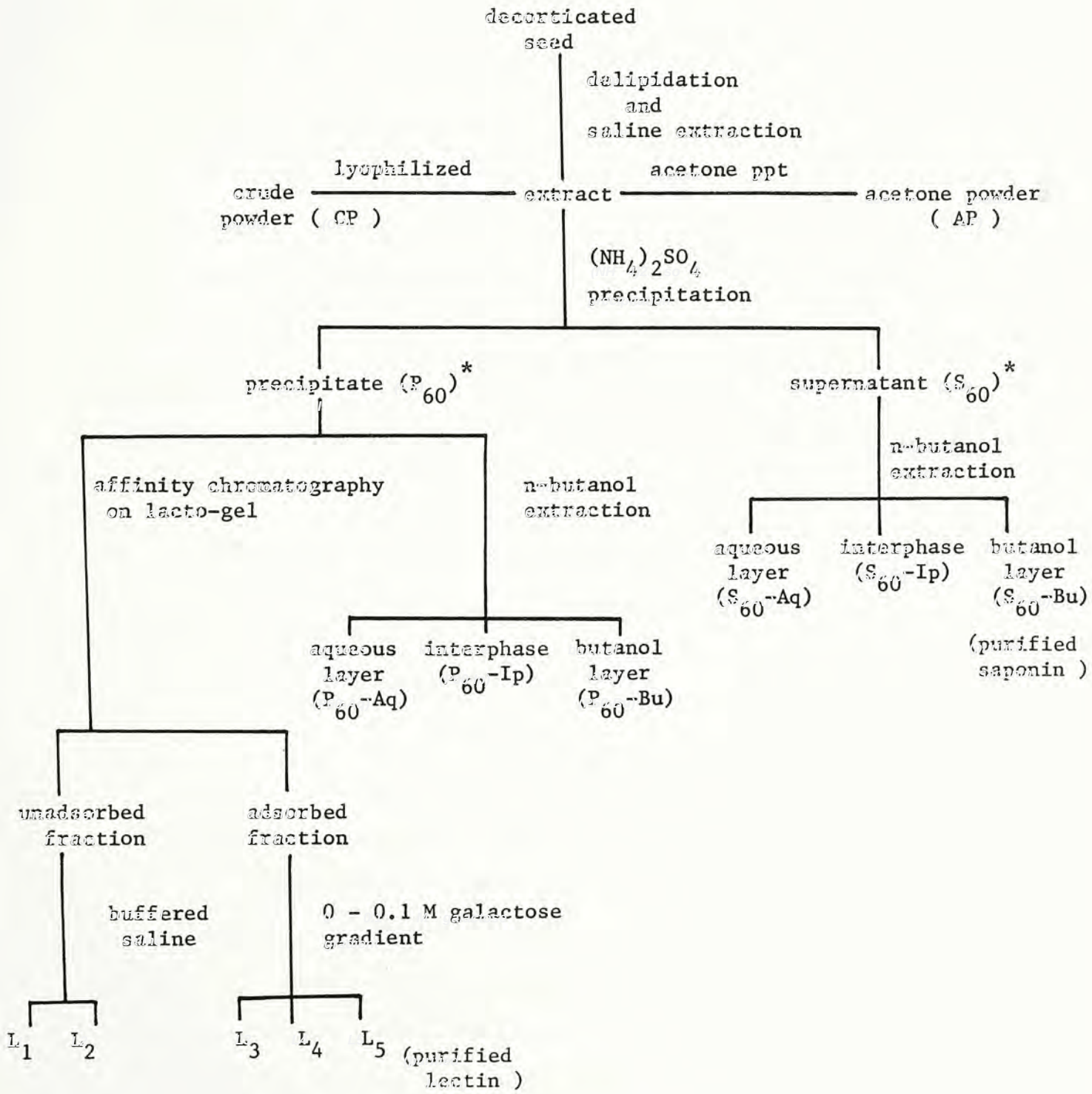
The purification steps of a D-galactose binding lectin from the seeds were outlined in Chart 1. In an isolation, 203g defatted powder and subsequently 14.4 g were obtained from 300 g of seeds. P_{60} was an intermediate partially purified fraction which could be further purified by lacto-gel chromatography to yield a number of fractions. A considerable amount of material was unadsorbed and divided into L_1 and L_2 fraction. The absorbed material was eluted by a linear concentration gradient of galactose and collected into three fractions labelled L_3, L_4 and L_5. L_5 is the highly purified lectin preparation. P_{60} was also fractionated by extraction with n-butanol to yield a P_{60}-Bu fraction (from the butanol layer), a P_{60}-Ip fraction (from the interphase) and a P_{60}-Aq fraction (from the aqueous layer). Hemolytic activity, an index of saponin activity, was absent from all fractions.

2.3.1b Saponin fraction

During the course of isolating D-galactose binding lectin from the seeds (Chart 1), a partially purified saponin fraction designated S_{60} was obtained. It could be further fractionated by extraction with n-butanol to yield a S_{60}-Bu

Chart 1

Purification protocol of a saponin and a D-galactose binding lectin from *Momordica charantia* L. seeds



* P_{90}, P_{95}/ S_{90}, S_{95} are precipitate/supernatant obtained after addition of $(NH_4)_2SO_4$ to 90% and 95% instead of 60% saturation.

fraction (from the butanol layer), a S_{60}-Ip fraction (from the interphase) and a S_{60}-Aq fraction (from the aqueous layer). Saponin(s) were present in S_{60} and S_{60}-Bu. No proteins were detected in S_{60}-Bu.

2.3.1c Abortifacient proteins

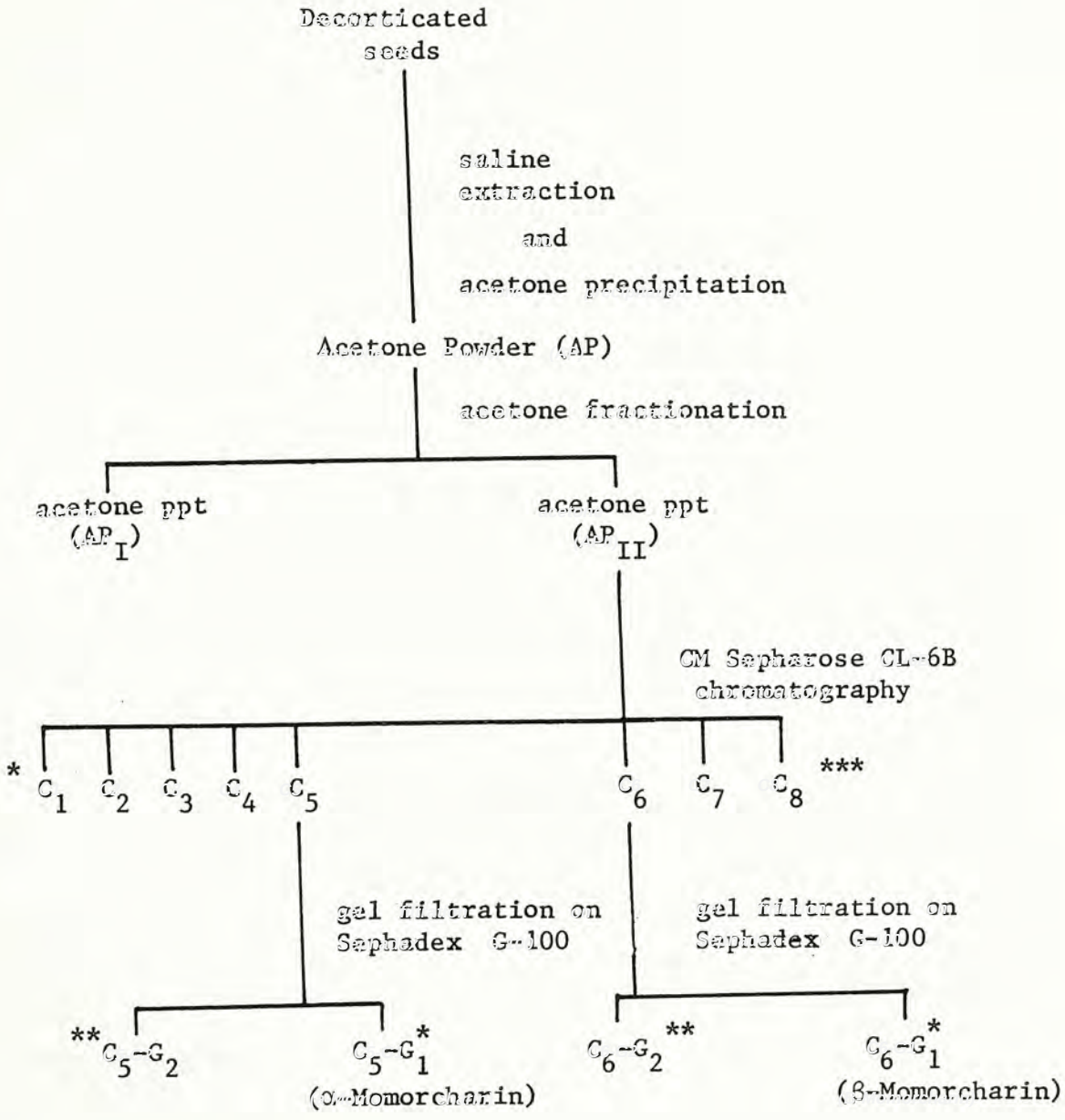
Briefly, for isolation of α-momorcharin and β-momorcharin, Momordica seeds were extracted with normal saline and the extract was subjected to acetone fractionation (Chart 2). The precipitate which formed was redissolved and subjected to chromatography on CM-Sepharose CL-6B (Pharmacia). Fractions eluted from the column successively with a salt gradient designated C_1, C_2, C_3, C_4, C_5 and C_6. C_5 and C_6 are the partially purified preparations of abortifacient proteins. Chromatography of C_5 and C_6 on Sephadex G-100 fine (Pharmacia) yielded α-momorcharin (= C_5-G_1) and β-momorcharin (= C_6-G_1) and the corresponding low molecular weight inactive contaminents C_5-G_2 and C_6-G_2.

2.3.1d Fruit and seed "p-peptide"

The method of Khanna and Jain (1981) was strictly followed in order to isolate p-peptide from the fruits as well as from the seeds of M. charantia (Chart 3) except that the step utilizing a 0.0001% zinc acetate solution to crystallize the peptide was deleted from the procedure, the reason being that zinc has been found to display some insulin-like activities in vitro (Coulston et al., 1980). The seed "p-peptide" was further purified by affinity chromatography on fetuin agarose.

Chart 2

Purification protocol of abortifacient proteins from *Momordica charantia* L. seeds (Law, 1983 ; Chan, 1984).



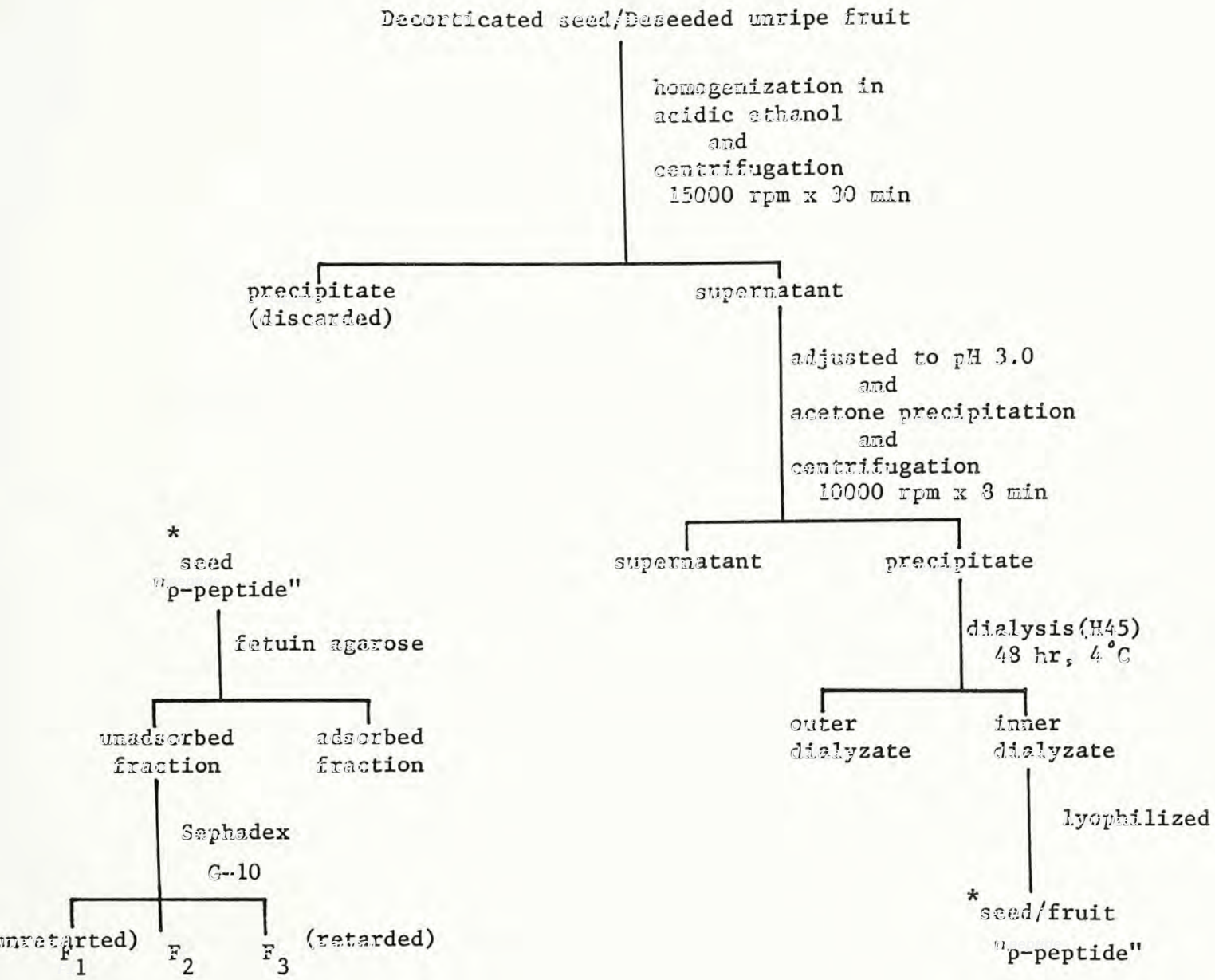
* : unretarded fraction

** : retarded fraction

*** : most retarded fraction

Chart 3

Purification protocol of "p-peptide" from *Momordica charantia* seeds and fruits (after Khanna *et al.*, 1981)



2.3.1e Insulin-like peptide by using the insect insulin purification scheme

The method of Leroith et al. (1980) was largely followed except that fraction S_4 obtained was further fractionated into seven fractions by CM Sepharose column chromatography (Chart 4).

2.3.1f Insulin-like-peptide by using the mammalian insulin purification scheme

According to Mirsky's method (1973) of extraction, if the starting material is bovine pancreas, fraction P_2 should be the purified insulin. But when this method was adopted to purify insulin-like peptide from M. charantia seeds, fraction P_2 was found to contain saponin activity such as hemolytic activity. So fraction P_1 was used instead and after CM-Sepharose CL-6B column chromatography, a number of fractions with different molecular nature were collected (Chart 5).

2.3.2 Heat treatment

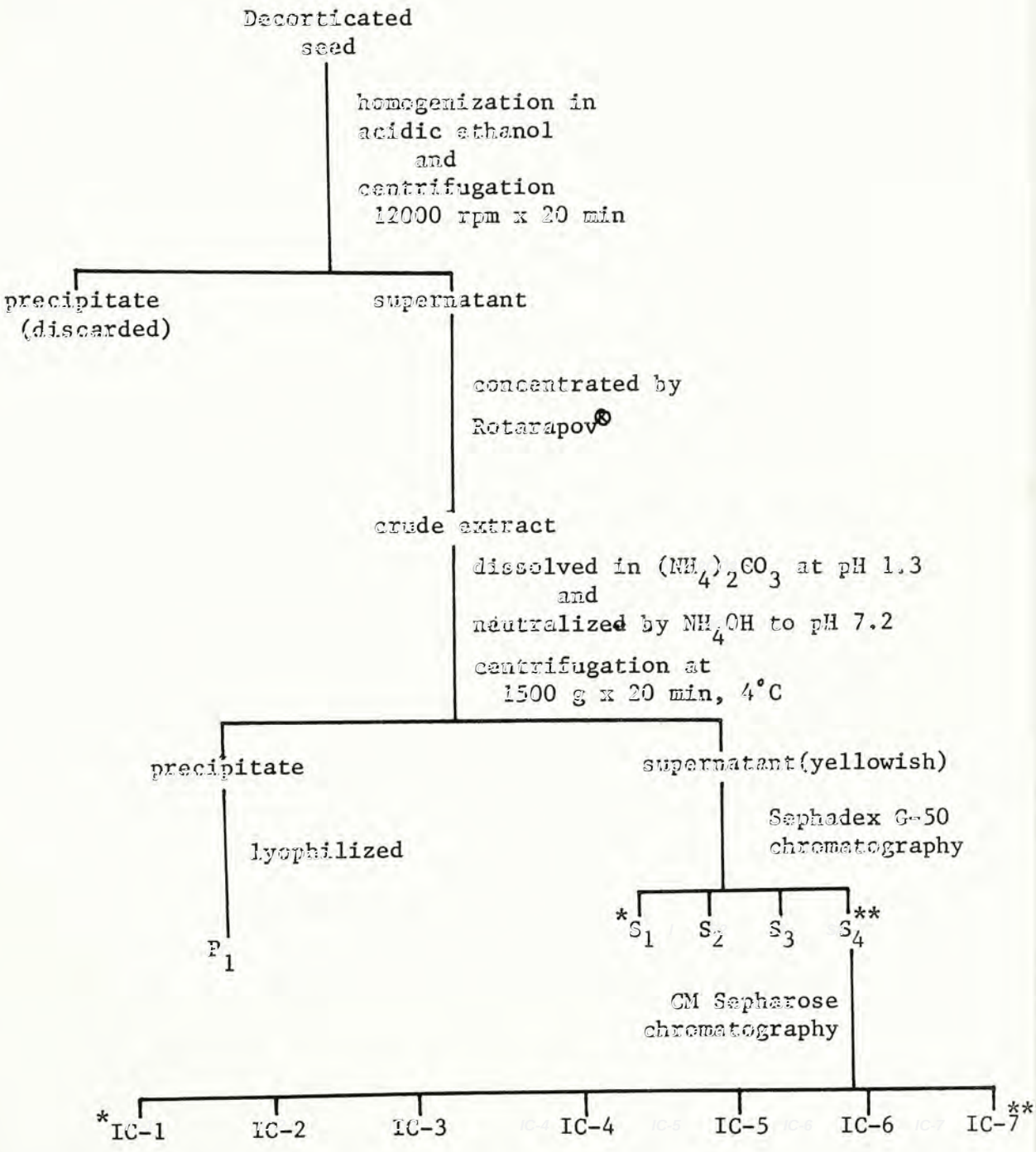
To investigate the heat lability of the various fractions, they were heated at 100°C for 15 minutes and then cooled down to room temperature prior to testing in the lipolysis or lipogenesis assay.

2.3.3 Enzyme treatment

Treatment of the fractions and the appropriate controls in ammonium acetate buffer with trypsin (at pH 8.0), Chymotrypsin (at pH 8.0), pronase E (at pH 7.5), emulsin (at pH 5.0) or

Chart 4

Extraction procedure of insulin-like fractions from *Momordica charantia* seeds (based on insect insulin purification scheme of LeRoith *et al.*, 1981)



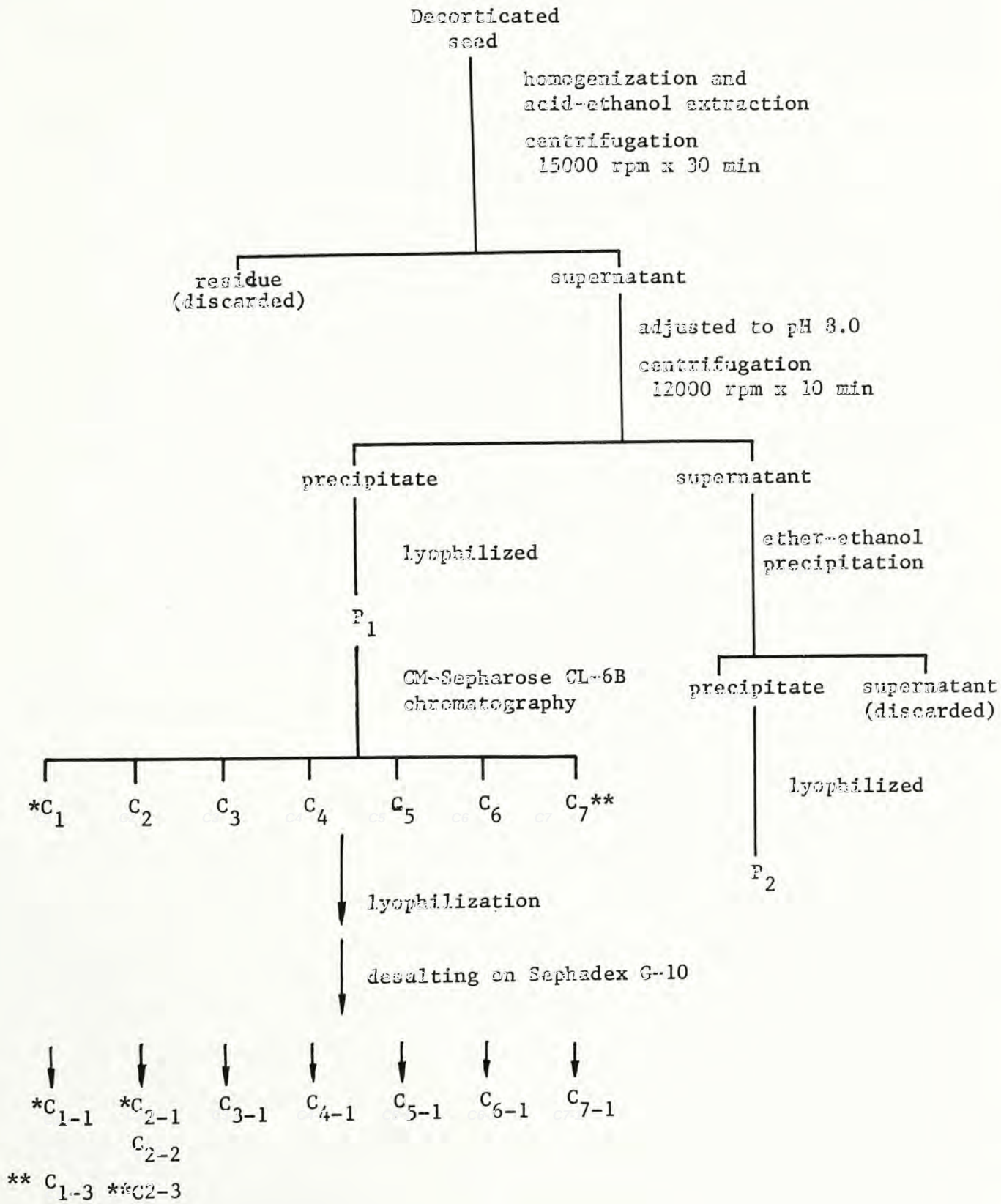
* : unretarted fraction

** : most retarded fraction

IC-3, IC-4 and IC-5 are insulin-like fractions

Chart 5

Extraction and purification of insulin-like peptide from *Momordica charantia* L. seeds (based on mammalian insulin purification scheme of Mirsky, 1973).



* : unretarded fraction

** : most retarded fraction

C_{1-3} and C_{6-1} are purified insulin-like fractions

glutathione (at pH 7.4) was carried out at an enzyme:substrate ratio of 1:50 (w/w) at 37°C for 5 hours. The reaction was stopped and the treated fractions and controls were then lyophilized to dryness prior to being assayed.

2.3.4 Hormone-/dbcAMP-induced lipolysis assay using rat adipocytes

2.3.4a Isolation of fat cells

Virgin male Sprague-Dawley rats (Charles River Laboratory, Japan), weighing 160-190 g and maintained on Purina Chow and water ad libitum, were sacrificed by cervical dislocation. Suspension of isolated fat cells were prepared by the method of Rodbell (1964) as modified by Gliemann (1967). Epididymal fat pads were carefully dissected out and placed in 0.9% saline. After blotting on filter paper, fat pads were cut with a sharp razor blade into strips about 2 mm across and placed in freshly prepared Krebs-Ringer bicarbonate (KRB) buffer (3 ml/g fat pad), containing half the amount of calcium suggested by Cohen (1951) and 2% bovine serum albumin (BSA), at pH 7.4 in Falcon polypropylene culture tubes. Collagenase (Sigma, Type II) was added at a ratio of 6 mg/g fat pad. The tubes were incubated in a Dubnoff metabolic water bath with shaking (90 cycles/min) at 37 °C for 75 min. At the end of digestion, the contents of the tubes were filtered throught two layers of cheesecloth into another culture tube. The filtrate was then centrifuged at 50 xg in a MSE GF-8 centrifuge for 2 min. The infranatant was aspirated and replaced with 10 ml fresh KRB containing 4% BSA.

After mixing, the tube was centrifuged at 50xg for 2 min. This washing procedure was repeated three times. The washed cells were resuspended in fresh KRB containing 4% BSA and 0.01% lima bean trypsin inhibitor. When 25 rats were used, it was possible to prepare 180 ml of adipocyte suspension containing 3-4 x 10^5 cells with a dry weight of 25-35 mg per ml of suspension. The cell suspension was then preincubated, in a 500 ml polypropylene beaker (Xlon, England) at 37°C under an atmosphere of 95% oxygen - 5% carbon dioxide for 1/2 hour, for the recovery of hormone receptors partially destroyed by trypsin present in crude collagenase (Kono, 1969).

2.3.4b Incubation of adipocytes with hormone/dbcAMP and fraction to be assayed

After preincubation, the fat cell suspension was gently stirred with a magnetic stirrer in order to ensure homogeneity. Using an autopipette (Gilson) equipped with a polypropylene tip which has been cut to make the orifice larger, one ml aliquots were rapidly transferred to polypropylene culture tubes (10x75 mm) each containing hormone and buffer or the fraction to be assayed in a total volume of 150 ul. When epinephrine bitartrate was used, ascorbate (0.025 mg/ml) was added to retard oxidation of the catecholamine (Schimmel, 1973). Incubation was performed at 37 °C under an atmosphere of 95% oxygen - 5% carbon dioxide with moderate shaking for two hours. At the end of incubation, 1 ml of 10% trichloroacetic acid (w/v) was added to each tube to stop the reaction. The tubes were centrifuged at 600xg in a CU-

5000 centrifuge (Damon) for 20 min.

2.3.4c Determination of Glycerol

Glycerol production at the end of the incubation, used as an index of lipolysis, was determined by the method of Lambert and Neish (1950) as modified by Ramachandran (1972). Supernatant (0.5 ml) was transferred from each tube with an autopipette, avoiding the fatty top layer, to a 15x150 mm glass tube containing 0.1 ml 1N sulphuric acid . Then 0.1 ml of 50 mM sodium periodate was added to each tube to oxidize glycerol. After 5 min, 0.1 ml of 10% sodium metabisulfite was added to reduce the excess periodate. After an additional 5 min, 3.3 ml of chromotropic acid reagent (1g 4,5-dihydroxynaphthalene-2,7-disulfonic acid disodium salt : 100 ml H_2O : 400 ml 12.5 M H_2SO_4) were added and the mixture was vortexed. The tubes were covered with marbles and placed in a boiling water bath for 30 min. One ml of water was then added. The tubes were vortexed and allowed to cool down to room temperature before absorbance at 570 nm was read with a Spectronic 21 spectrophotometer (Bausch & Lomb).

2.3.4d Determination of fat cell dry weight

Six one-ml aliquots of fat cells were filtered through preweighed wetted glass fibre filters (Whatman GF/C). The filters were desiccated overnight in a lyophilizer. Filters without fat cells were similarly desiccated. Fat cell dry weight was then calculated from the weight difference between the two types of filters.

2.3.5 Hormone-induced lipolysis assay using hamster adipocytes

Virgin male golden hamsters (animal house, University of Hong Kong), weighing 120-140g, were killed by cervical dislocation. Both epididymal and perirenal fat tissues were used. The procedure for isolation of hamster fat cells was essentially the same as that described above for rat adipocytes except that the digestion time was reduced to 45 minutes and the shaking speed to 60 cycles/min. During dispersion of fat cells, the cell suspension was kept homogeneous by swirling. The procedures for adipocyte incubation with hormone and assay fractions, glycerol determination, and adipocyte dry weight determination, were similar to those described above for rat adipocytes.

2.3.6. Lipogenesis assay using rat adipocytes

Lipogenesis was determined by measuring the incorporation of D-(3-3H)-glucose into lipids as described by Moody et al. (1974). Fat cells were prepared from epididymal fat pads of Sprague-Dawley rats (160-190 g) as described in the rat adipocyte lipolysis assay except that the cells were washed and diluted in KRB buffer containing 0.55 mM glucose and 10 mg/ml BSA. Cell suspension (120 ml) containing 4-8 mg of lipid/ml could be prepared from six rats.

2.3.6a Incubation and lipid extraction

The fat cell suspension was kept homogeneous by gentle stirring with a magnetic stirrer. Aliquots (0.9 ml) were added to

plastic counting vials containing 0.1 μCi of D-(3-3H)-glucose in 20 μl and 0.1 ml of insulin standard or fraction to be assayed. The vials were incubated at 37°C under an atmosphere of 95% oxygen-5% carbon dioxide with moderate shaking for two hours in a Dubnoff metabolic incubator. At the end of incubation, 0.2 ml of 8N sulfuric acid was added to terminate the reaction. Total lipid of adipocytes was extracted by shaking with 15 ml toluene-based scintillant containing 0.5% PPO(w/v) and 0.03% dimethyl POPOP(w/v), left at room temperature for 1 hr and then counted in a Beckmann LS 7000 scintillation counter. Blanks were prepared by adding cells to aliquots of the D-(3-3H)-glucose used and stopping the incubation immediately. The counts recorded were corrected for the scintillant background. The total counts added to each vial were determined by adding 14.6 ml of toluene : triton X-100 (2:1) scintillant containing 0.4% (w/v) PPO and 0.02% (w/v) POPOP.

2.3.6b Determination of total cellular lipids

Total cellular lipids were determined by the method of Dole and Meinertz (1960). One ml of cell suspension was extracted with five ml 2-propanol-heptane (4:1) in a 15 ml glass-stopped tube (Corning) with mild shaking and then allowed to stand at room temperature for 5 min followed by the addiion of 3 ml heptane and 2 ml water. After shaking, the system was allowed to stand for separation into two phases. Aliquots (0.5 ml) of the heptane phase in a 15 ml glass tube were blown down to dryness with nitrogen gas. Large amounts of fat cells were prepared, and the total lipids, extracted fom the cells by the method described

above, were used as standard. Briefly, 200.0 mg of solvent-extracted epididymal adipocyte lipids were added to a volumetric flask and diluted to 100 ml with absolute ethanol. Various volumes (0.1 ml, 0.2 ml, 0.3 ml and 0.4 ml) of this standard were added to glass tubes and blown down to dryness with nitrogen gas. Lipid was quantitated photometrically by the sulfo-phospho-vanillin reaction (Frings et al., 1972). Concentrated sulfuric acid (0.2 ml) was added to the standard and unknowns and the tubes were well vortexed. All tubes were placed in boiling water for 10 min(± 1 min) and cooled in cold water for about 5 min. Ten ml phospho-vanillin reagent containing 0.6% vanillin : water : concentrated phosphoric acid (7 : 1 : 12) were then added and mixed well before incubation at 37°C(± 2°C) in a water bath for 15 min. The tubes were cooled for about 5 min before absorbance at 540 nm was measured within 30 min with a Spectronic 21 spectrophotometer (Bausch & Lomb).

2.3.7 Monitoring of plasma glucose and total lipid of aged rats

2.3.7a Animal

Male Sprague-Dawley rats, 21-22 months of age, were raised under the same conditions of diet, lighting, temperature and humidity control. The animals were fed on Purina Chow which was relately low in fat (4%).

2.3.7b Blood sampling

All studies began at about 1100 hr in order to minimize variations caused by circadian rhythms. The rats were subjected

to a 24 hr fast prior to the beginning of each experiment. Pentobarbital (45 mg/Kg) was injected intramuscularly 30 min before the first collection of blood. Insulin or fractions in a volume of about 0.25 ml were injected subcutaneously. Blood was obtained from the tail at hourly intervals after drug injection. The tail tip was firstly rubbed with 75% ethanol(w/v) and cut with a pair of scissors but not so deeply as to strike any bone. One drop of blood was gently milked out and discarded in a paper towel. Approximately 0.5 ml of blood was collected in a 1.5 ml Eppendorf polypropylene vial containing 5 µl heparin solution (10 mg/ml saline). Blood cells were immediately spun down and plasma was aliquoted into tubes for plasma glucose and total lipid determination. Subsequent blood samples could be obtained by merely rubbing away clots on the wound with a paper towel soaked with 75% ethanol. During the experiment, the anaesthesized rats were kept warm with towels wrapped round their bodies and a table lamp above them. One ml saline was injected intraperitoneously to replenish water loss. Additional doses of pentobarbital (about 1/3 of the first dose) were required to keep the rats in the anaesthesized state.

2.3.7c Determination of plasma glucose

Plasma glucose concentration was determined by the glucose oxidase method as described by Keston (1956) and modified by Raabo Terkildsen (1960). Five ml of combined enzyme-color reagent solution containing glucose oxidase (500 IU/100 ml) and 0.25% O-dianisidine dihydrochloride (w/v) were added to tubes

containing 25 µl plasma and 0.5 ml of water. Each tube was mixed thoroughly before incubation at 37°C for 30±5 min. At the end of incubation, absorbance was measured at 440 nm within 30 min.

2.3.7d Determination of total plasma lipids

Total plasma lipid concentration was measured by the sulfo-phospho-vanillin reaction as described above, using olive oil as standard. Twenty µl of plasma were used in each determination.

2.4 Results and Discussion

2.4.1 Establishment of the assay systems

2.4.1a Lipolysis assays using rat adipocytes

Glycerol was used instead of fatty acid release as an index of lipolysis because glycerol, unlike fatty acids, does not reesterify after lipolysis (Patten, 1970). Colorimetric determination of glycerol with chromotropic acid reagent gave a high degree of accuracy and reproducibility for measuring glycerol production in lipolysis assay. Figure 2-1 shows the relationship between absorbance at 570 nm and concentration of glycerol used as the standard. The chief advantages of the method are simplicity and sensitivity. The colour developed is linear up to 0.2 μmole glycerol per tube assayed and is stable for at least 2 hours at room temperature.

Figure 2-2 shows a typical dose-response curve of epinephrine bitartrate-induced lipolysis in rat adipocyte. To prevent air oxidation to catechrome, epinephrine bitartrate aliquots were prepared in ascorbate solution about 15 minutes before the commencement of the assay. Unless otherwise stated, a submaximal dose of epinephrine bitartrate (0.33 μg/tube) was used to provide the lipolytic stimulus. The antilipolytic activity of a sample was determined by measuring adipocyte glycerol production in the presence of both epinephrine and the sample. Propranolol, a β-adrenergic receptor blocker, inhibited epinephrine-induced lipolysis in a dose dependent manner (Figure 2-3).

Figure 2-4 shows a typical dose dependent lipolytic response of rat adipocytes to corticotropin. Unless otherwise

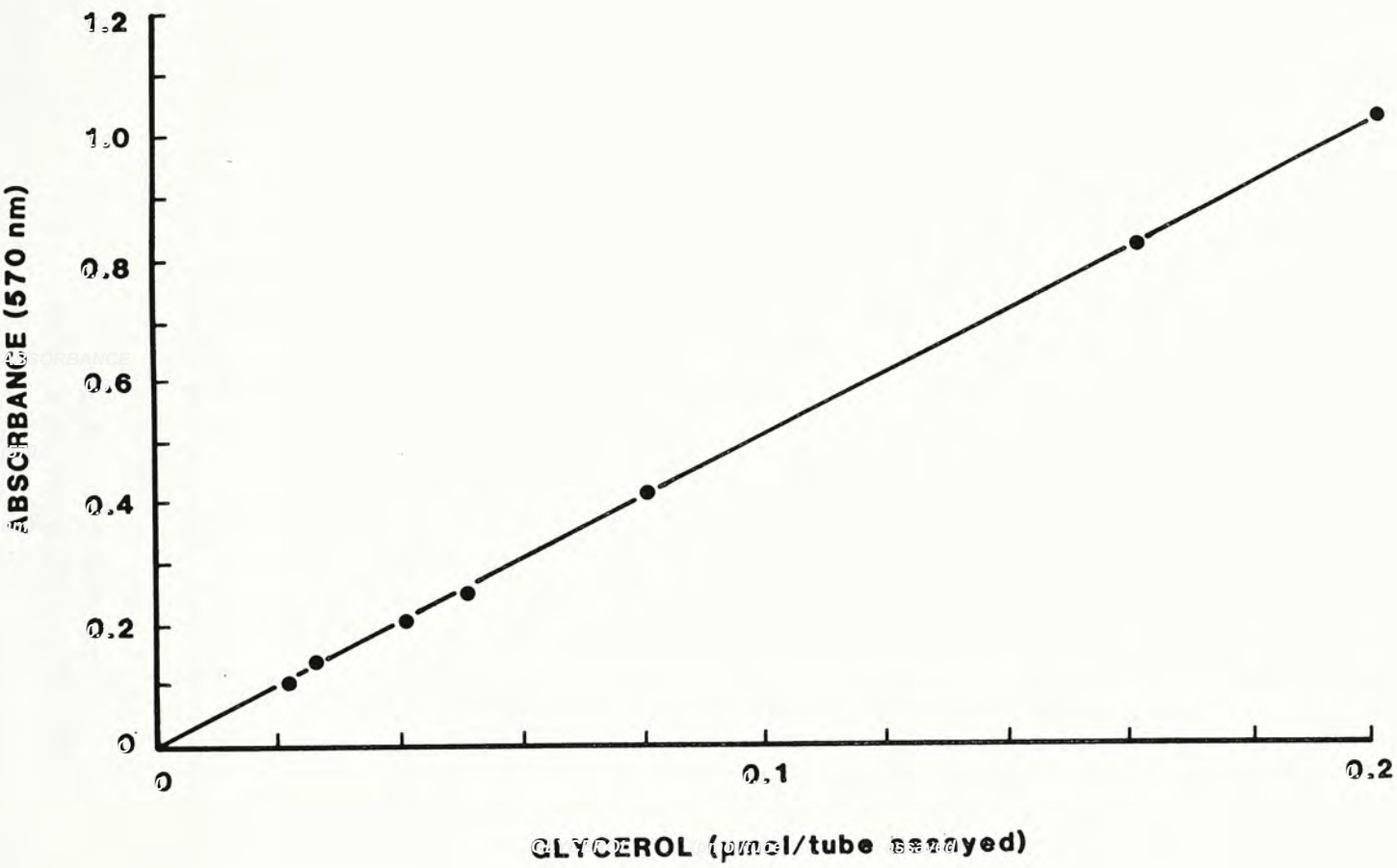


Figure 2-1. Standard curve of glycerol determination. Each point is the mean of triplicates.

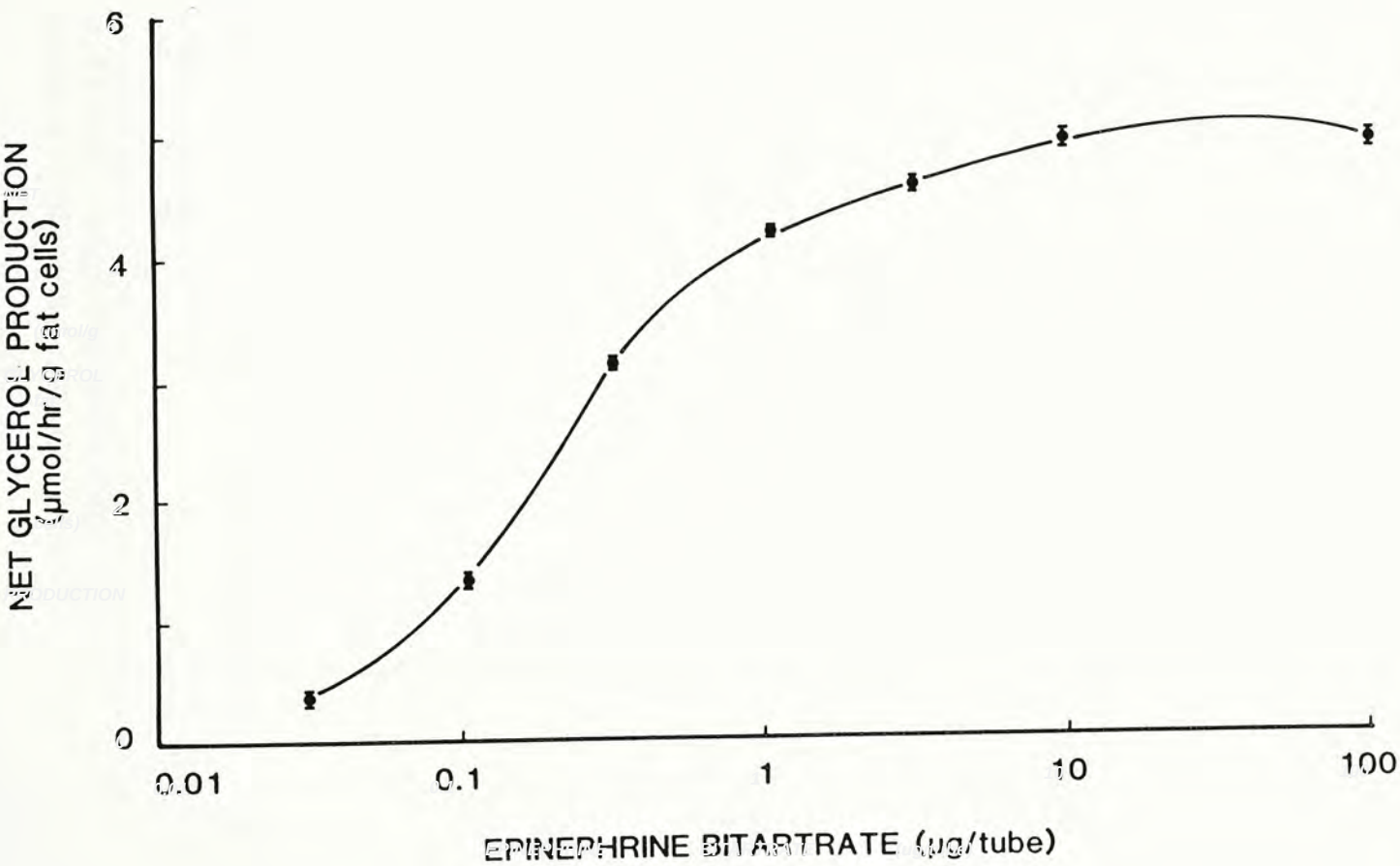


Figure 2-2. A typical dose response curve of epinephrine bitartrate-induced lipolysis in rat adipocytes. A submaximal dose (0.33μg) was chosen to induce lipolysis in antilipolysis assays of samples.

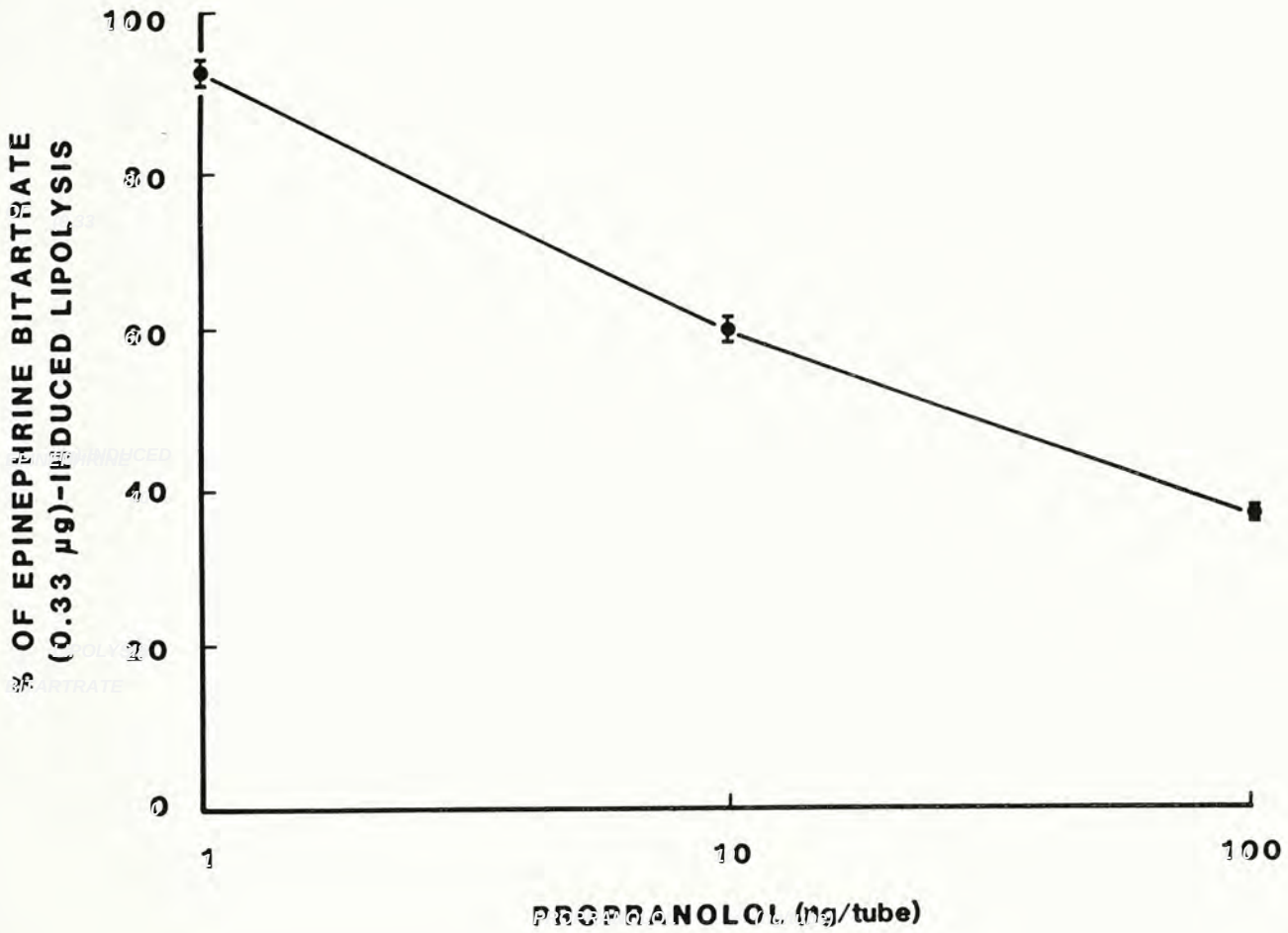


Figure 2-3. Effect of propranolol on epinephrine-induced lipolysis. Propranolol and epinephrine bitartrate (0.33µg) were added to fat cells at the same time. The glycerol released was expressed as percentage of glycerol production due to epinephrine bitartrate (0.33µg) alone.

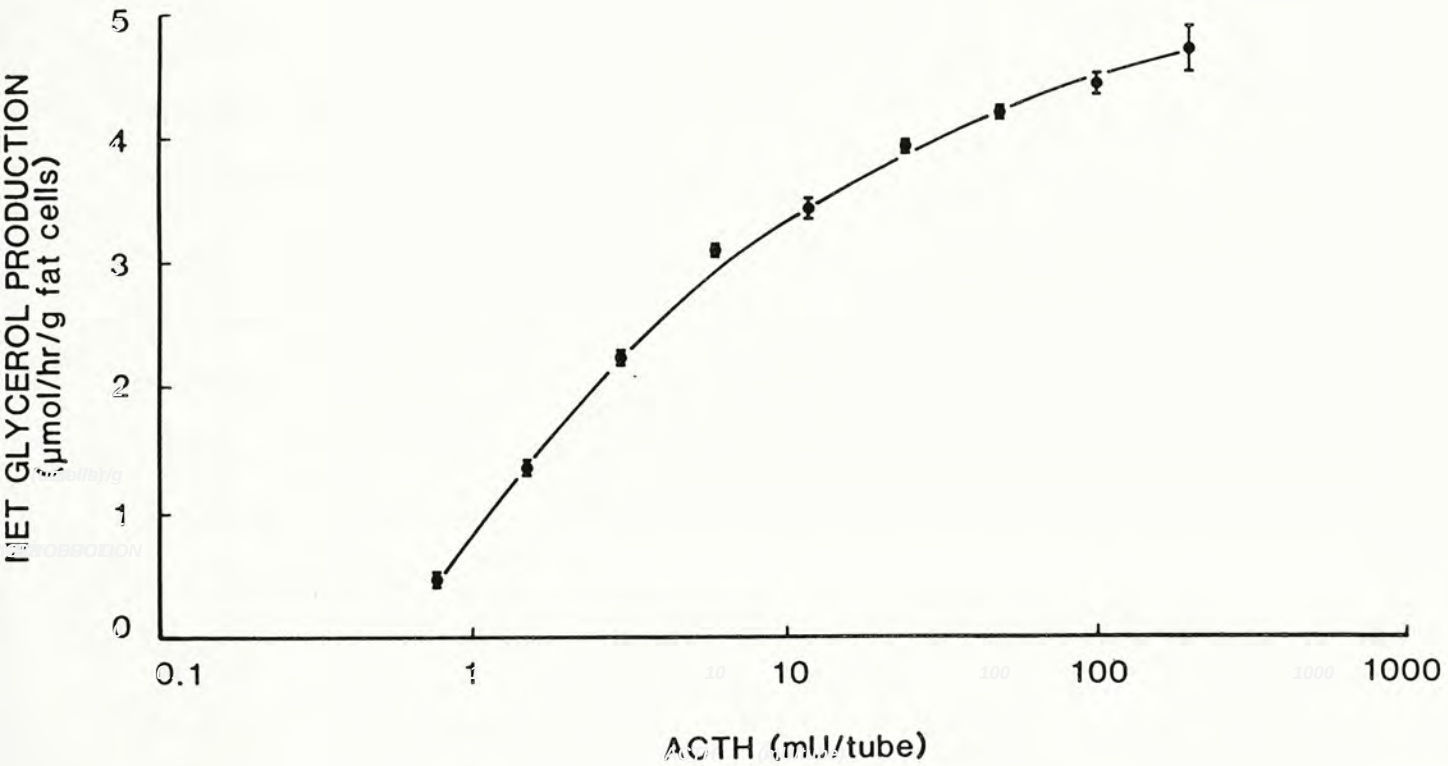


Figure 2-4. A typical dose response curve of ACTH-induced lipolysis in rat adipocytes. A submaximal dose (25mU/tube) was chosen to induce lipolysis in antilipolysis assays of samples.

stated, submaximal dose of corticotropin (25 mU/tube) was used throughout the study to stimulate lipolysis in rat adipocytes in the assay for antilipolytic activity. Propranolol also inhibited corticotropin-induced lipolysis in dose-dependent manner (Figure 2-5); although it was a better inhibitor of epinephrine-induced lipolysis.

The dose-dependent lipolytic response to glucagon was presented in Figure 2-6. A submaximal dose of glucagon (0.2 ug/tube) was used throughout the study to stimulate lipolysis in rat adipocytes in the assay for antilipolytic activity.

As already mentioned in introduction, the mechanism of epinephrine-, norepinephrine-, corticotropin-, and glucagon-induced lipolysis lies in the activation of adipocyte adenylate cyclase which increases the intracellular cAMP level and hence triggers a cascade of reactions leading to the breakdown of triglycerides into fatty acids and glycerol. However, even when a compound is found to be able to suppress hormone-induced lipolysis, we still do not know whether its antilipolytic activity results from an interaction with the lipolytic hormone, an interaction with the hormone receptor, or an effect on adenylate cyclase, phosphodiesterase or one of the enzymes beyond the cAMP level. If dbcAMP instead of hormone is used as the lipolytic agent, we can have a better understanding of the mechanism of an antilipolytic agent. Figure 2-7 shows a typical dose-response curve of dbcAMP-induced lipolysis. A maximal dose (2 mM) was chosen to induce lipolysis in the assay for antilipolytic activity.

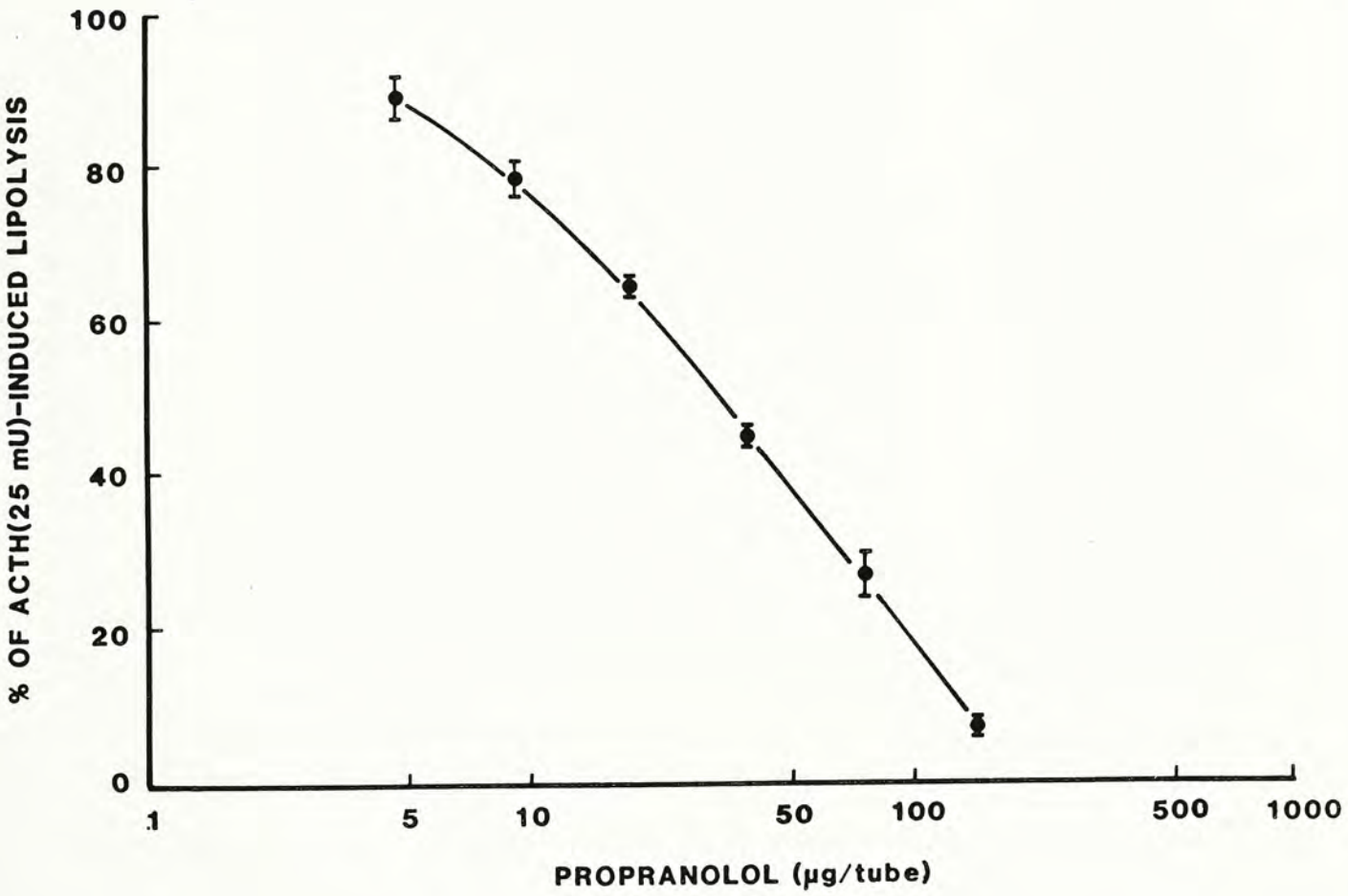


Figure 2-5. Effect of propranolol on ACTH-induced lipolysis. Propranolol and ACTH (25mU) were added to rat adipocytes at the same time. The glycerol released was expressed as percentage of glycerol production due to ACTH (25mU) alone.

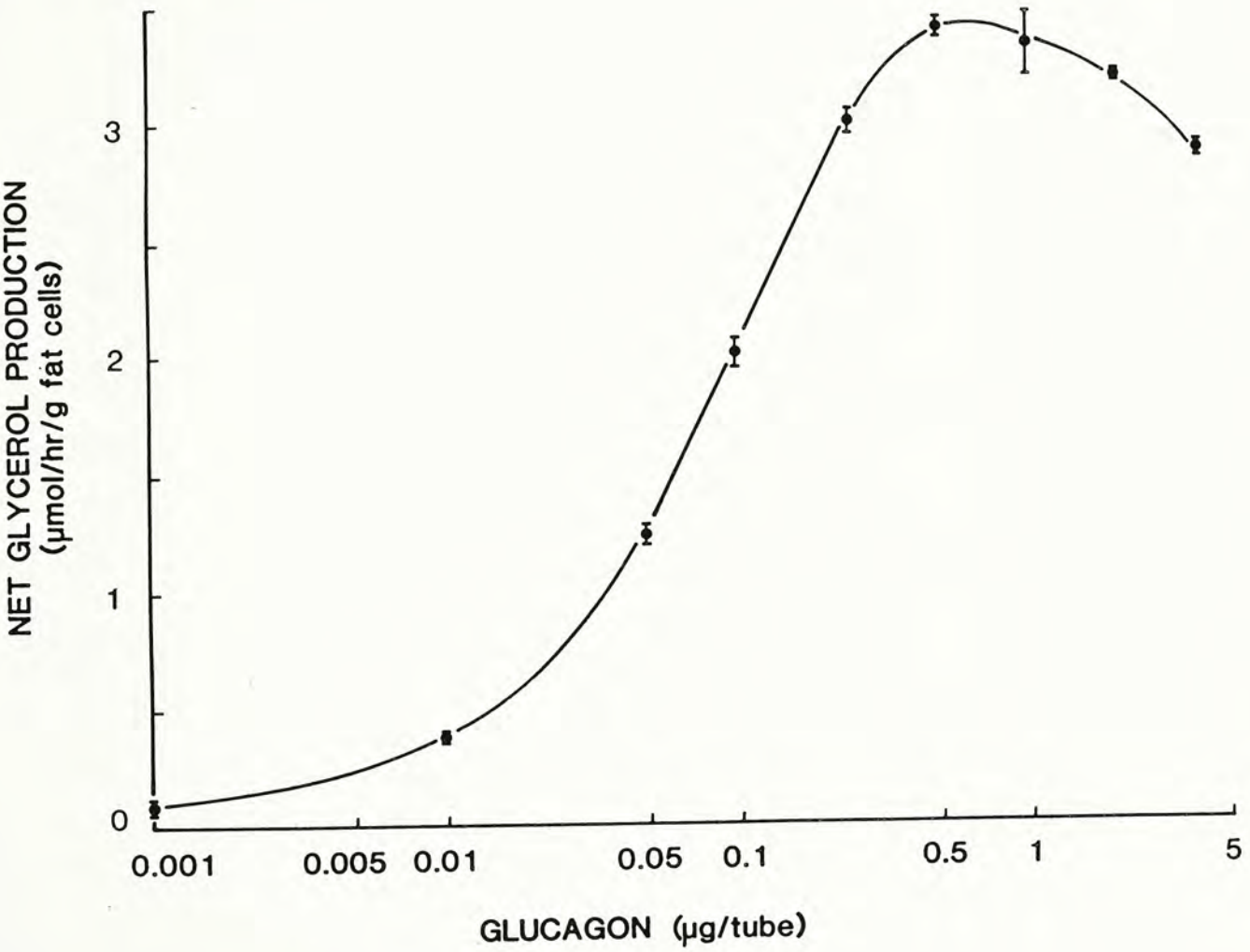


Figure 2-6. A typical dose response curve of glucagon-induced lipolysis in rat adipocytes. A submaximal dose (0.2µg/tube) was chosen to induce lipolysis in antilipolysis assays of samples.

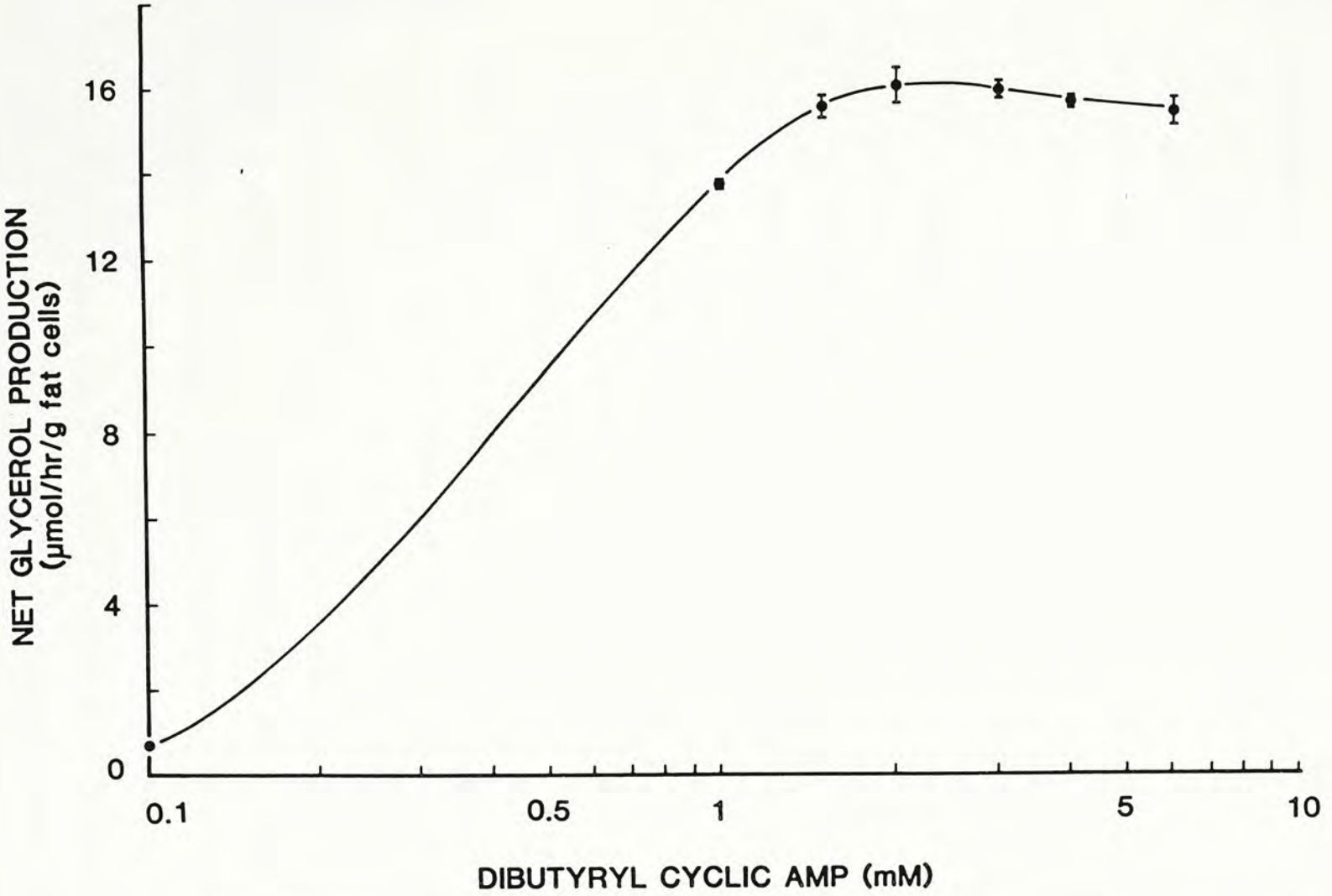


Figure 2-7. A typical dose response curve of dbcAMP-induced lipolysis in rat adipocytes. A maximal dose (2 mM) was used to induce lipolysis in order to investigate the mechanism of an agent which suppressed hormone-induced lipolysis.

2.4.1b Lipolysis assay using hamster adipocytes

The lipolytic response of hamster adipocytes (both epididymal and perirenal) to corticotropin was greater than that of rat epididymal adipocytes. One milliunit corticotropin stimulated lipolysis maximally (Figure 2-8). A submaximal dose of 0.5 mU/tube corticotropin was therefore used to induced lipolysis when hamster adipocytes were used.

2.4.1c Lipogenesis assay

Lipogenesis assay, a simple and reliable bioassay for insulin-like activity, is based on the incorporation of tritiated glucose into the lipid of isolated rat adipocytes. The blank counts for tritiated glucose plus scintillator are very low and the toluene-based scintillator efficiently extracts and counts the cellular total lipids (Moody et al., 1973). Figure 2-9 shows the dose response curve of insulin in stimulating the incorporation of tritiated glucose into total lipids. The net incorporation was expressed as percentage of the total count added per 10 mg total lipids. The use of tritiated glucose offers the advantage that tritium in glucose is counted at a very low efficiency at the interphase and that direct extraction of adipocyte total lipids into the scintillant in the counting vial and subsequent radioactivity counting can be carried out. No radioactive carbon dioxide evolves during the incubation. (3-3H)-Glucose was used since it was incorporated more efficienctly into total lipids than (2-3H) and (6-3H) glucose. The assay

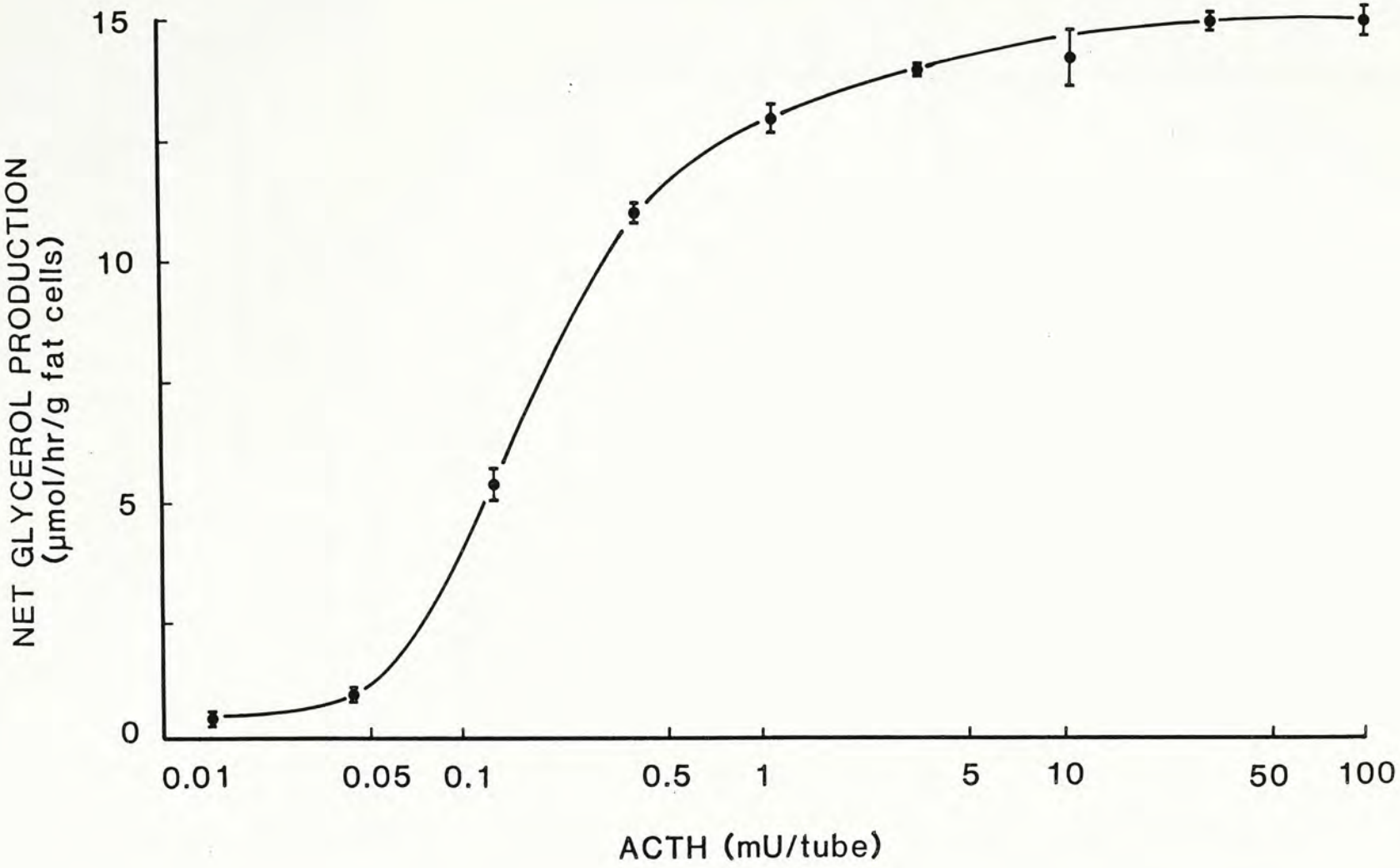


Figure 2-8. Dose response curve of ACTH-induced lipolysis in hamster adipocytes. A submaxmal dose of 0.5 mU/tube was used to induced lipolysis in antilipolysis assays of samples.

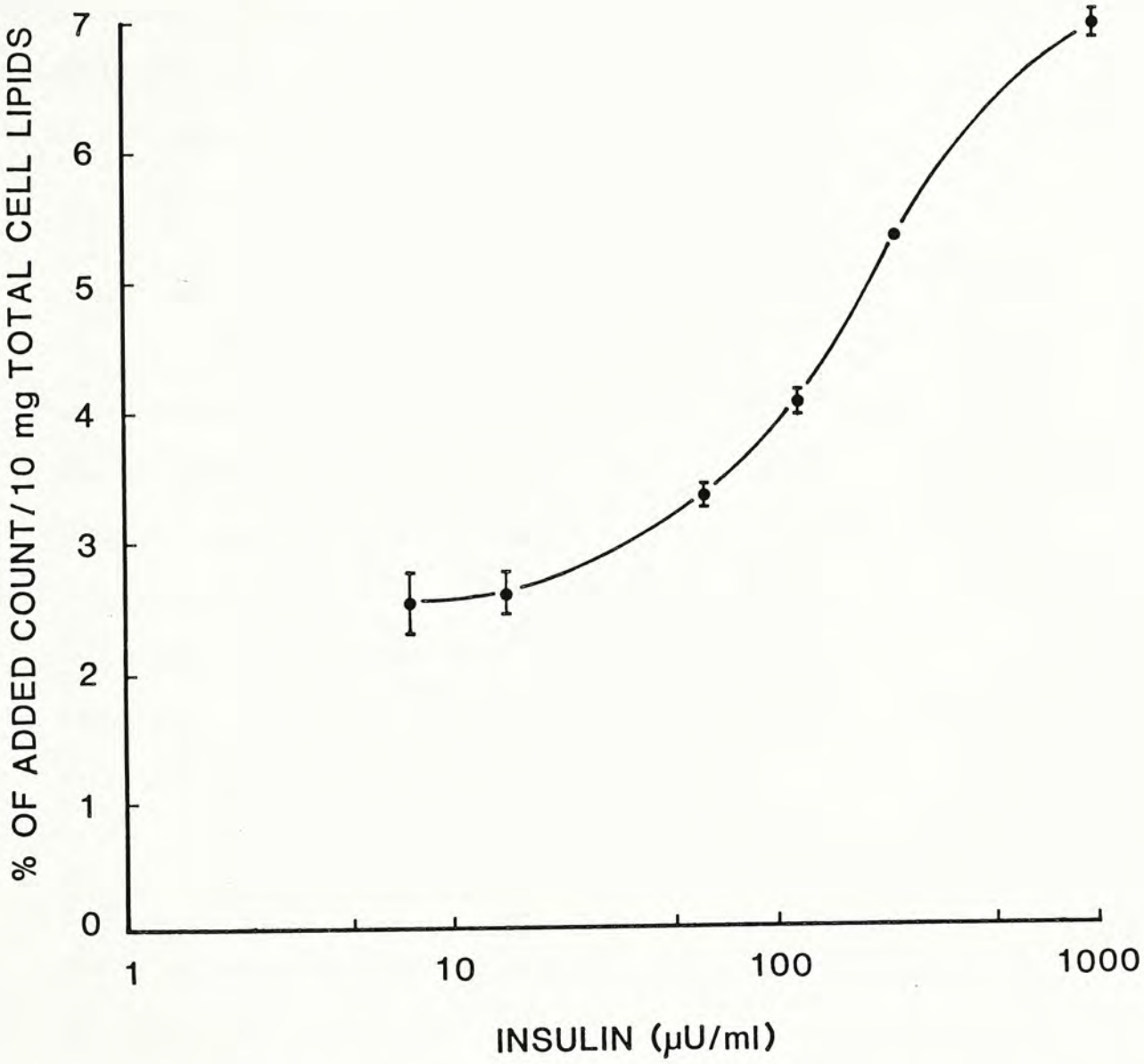


Figure 2-9. Dose response curve of insulin stimulated lipogenesis from D-[3-3H]-glucose into lipid. The net incorporation of tritiated glucose was expressed as percentage of total counts added per 10 mg total lipids.

technique had been used for measuring insulin-like compounds.

Figure 2-10 shows the standard curve for the colorimetric determination of adipocyte total lipids by the sulfo-phospho-vanillin reaction. The linearity of the assay is up to 1 mg.

2.4.2 M. charantia lectin and its chromatographically related fractions

In a preliminary study to detect the presence of insulinomimetic substance(s) in M. charantia seeds, both crude powder (CP) and acetone powder (AP) were (Chart 1) found to inhibit corticotropin-stimulated and epinephrine-stimulated lipolysis. The drastic reduction (Table 2-1) in the antilipolytic activities after heat treatment indicated the existence of a proteinaceous factor with some insulinomimetic activity.

The antilipolytic activities of the fractions derived from P_{60} were presented in Figure 2-11. It can be seen that all fractions had lower activity than P_{60}. The dose response of P_{60}-Ip was parallel to but shifted to the right of that of P_{60}, suggesting denaturation of the antilipolytic activity by n-butanol. P_{60}-Aq had minimal activity. The activity in L_5, P_{60}, and P_{60}-Ip were all abolished after heat treatment (Figure 2-12).

The antilipolytic activity of P_{60} was concentrated in L_5. L_{1-2}, a mixture of L_1 and L_2, had undetectable activity at the dose (100 µg) tested. There was not enought L_3 available for assay. The activity in L_4 could be attributed to the presence of L_5 in the fraction (Table 2-2). It is worth noting that the

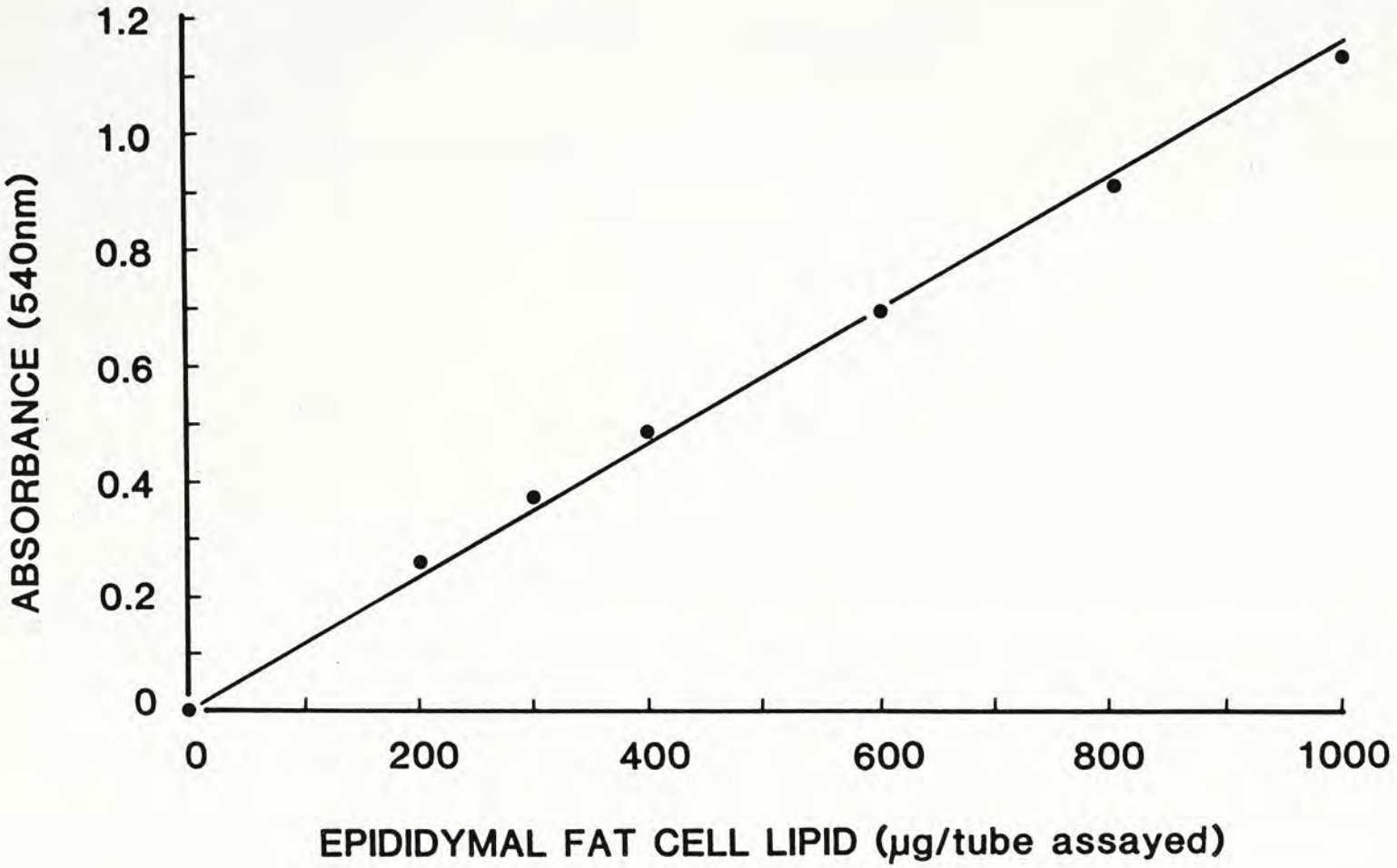


Figure 2-10. Standard curve of total adipocyte lipid determination. A large amount of total cell lipids was extracted from isolated rat adipocytes and used as standard. The amount of lipid containedin the fat cells added to an incubation vial in the lipogenesis assay can be determined colorimetrically as described in the Method section.

Antilipolytic activities of CP and AP and effect of heat treatment

Experiment No.	Fraction	Dose (µg/tube)	Lipolysis		
			% of activity of 25 mU ACTH	% of activity of 0.33 µg epinephrine bitartrate	
				No heat treatment	Heat treatment
1	CP	500	68.3 ± 2.3		
		250	87.6 ± 2.6	—	—
		50	109.0 ± 6.5		
2	AP	500	61.2 ± 2.1		
		250	87.7 ± 0.5		
		50	94.1 ± 0.9		
3	AP	1000		43.5 ± 0.9	
		500	—	60.2 ± 2.0	—
		250		76.9 ± 1.0	
4	AP	500	—	53.0 ± 0.8	91.3 ± 0.9

CP = crude powder and AP = acetone powder of *M. charantia* seeds

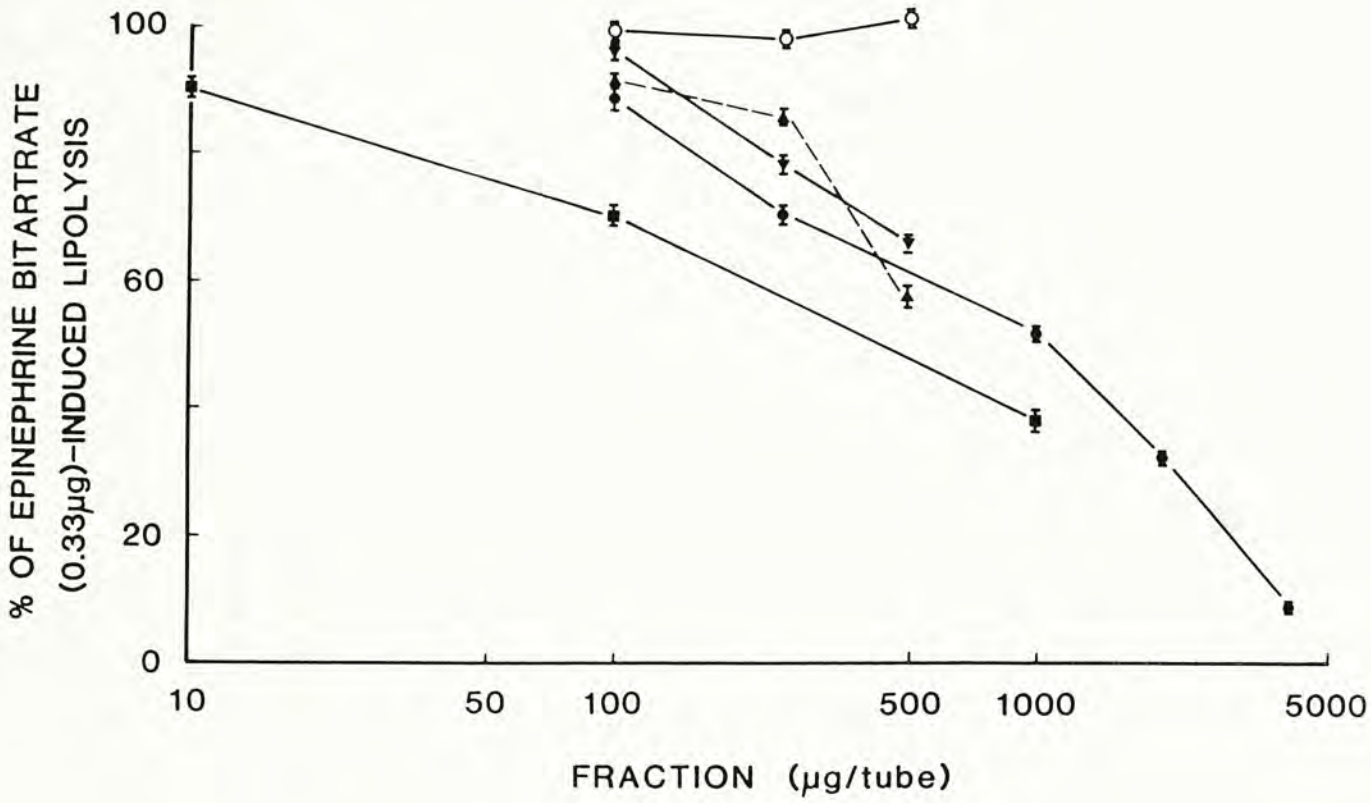


Figure 2-11. Effects of fractions derived from P_{60} on epinephrine-induced glycerol production. The dose response of P_{60}-Ip(▼) was parallel but shift to the right of that of P_{60}(●), implying loss of activity after n-butanol extraction. The activity of P_{60}-Bu (▲) might be due to the presence of a saponin detected by haemolysis test. P_{60}-Aq (○) had minimal activity. The dose response of propranolol (■) was parallel to that of P_{60}, suggesting that the antilipolytic component in P_{60} may act on receptor level.

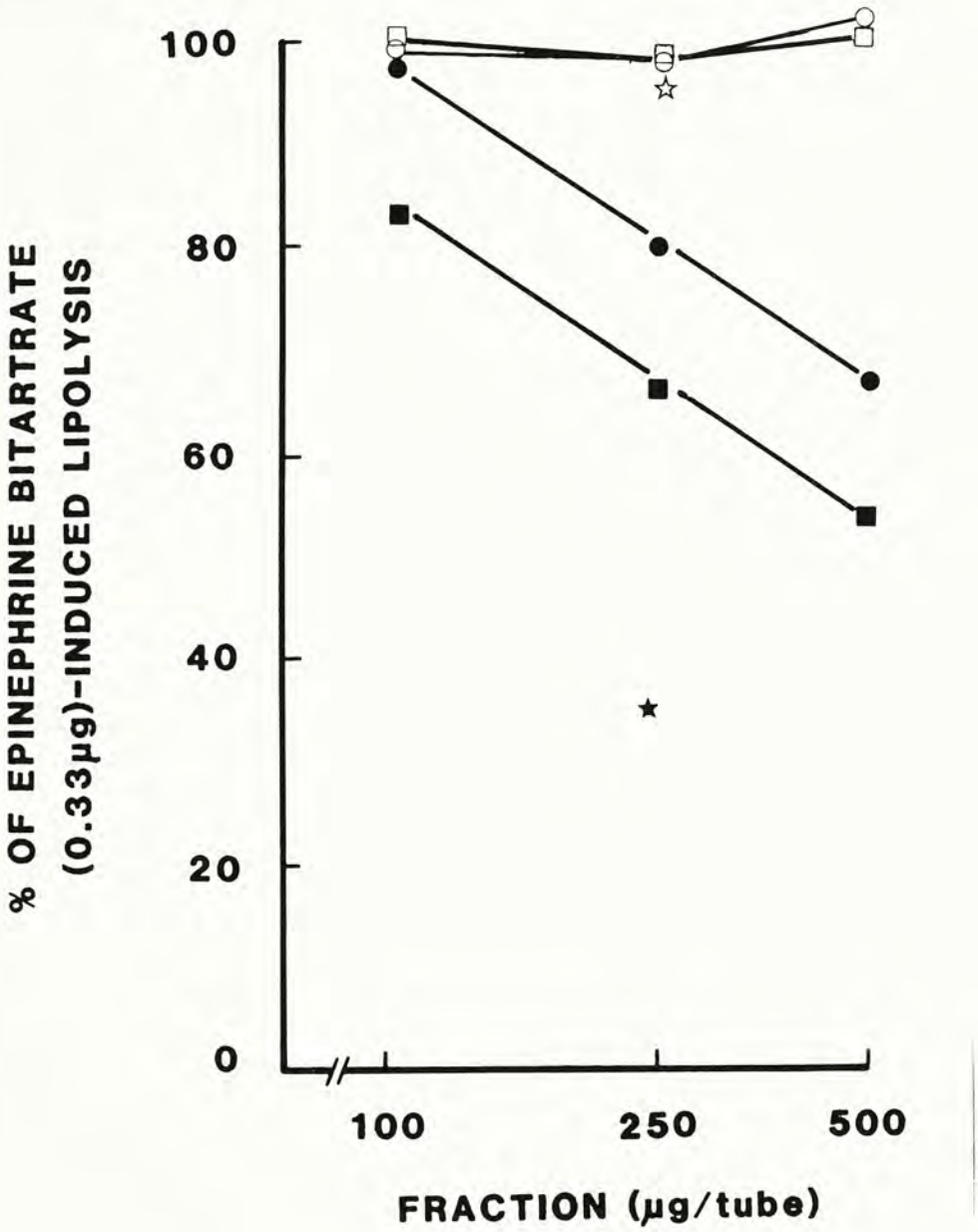


Figure 2-12. Effect of heat treatment on the antilipolytic activity of L_5 and P_{60}. The activities in L_5 (★), P_{60}-Ip (■) were all destroyed after heat treatment. (☆) heat-treated L_5. (○) heat-treated P_{60}. (□) heat-treated P_{60}-Ip.

Table 2-2

Antilipolytic activities of fractions derived from P_{60} after affinity chromatography on lacto-gel

Fraction	Dose (µg/tube)	% of 0.33 µg epinephrine bitartrate-induced lipolysis
None	-	100
L_{1-2}	100	98.1 ± 1.2
L_4	100	60.2 ± 0.6
L_5	100	44.7 ± 0
L_5	50	55.3 ± 7.8

Epinephrine bitartrate (0.33 µg) elicited an increase of glycerol production over the control at the rate of 2.64 ± 0.03 µmol glycerol/hr/g fat cell dry wt.

inhibition curves of P_{60} and L_5 in the lipolysis assay were parallel to each other and to that of propranolol (Figure 2-13). When a dose of L_5 above 10 µg was tested, the dose dependency disappeared (Figure 2-13) probably because of considerable aggregation of adipocytes induced by the lectin which could be observed microscopically (Figure 2-14). Such surface phenomenon was also observed in the presence of P_{60} (Figure 2-15).

The antilipolytic activity in P_{60}, similar to that in L_5, decreased after treatment with trypsin, chymotrypsin, glutathione and galactose but retained after exposure to α-methyl-D-glucoside (Table 2-3).

When tested at various doses ranging from 2 to 500 µg L_5 did not manifest any intrinsic lipolytic activity (Table 2-4). The lipogenic activity of L_5, in comparison with that of bovine insulin, is presented in Figure 2-16.

Both P_{60}, a partially purified lectin preparation (Figure 2-17a) and the lectin (Figure 2-17b), produced an antilipolytic effect which could be overcome by increasing the dose of epinephrine, the lipolytic agent. A similar phenomenon was observed when glucagon provided the lipolytic stimulus instead of epinephrine (Figure 2-18). However, raising the concentration of corticotropin was ineffective in removing the inhibitory action of the lectin on corticotropin-induced lipolysis (Figure 2-19). A time course study on the antilipolytic effect of P_{60} revealed that the effect was reversible to a certain extent by the addition of either epinephrine or corticotropin 1 hr 45 min after the commencement of the assay (Figure 2-20a and 2-20b) which was only 15 minutes

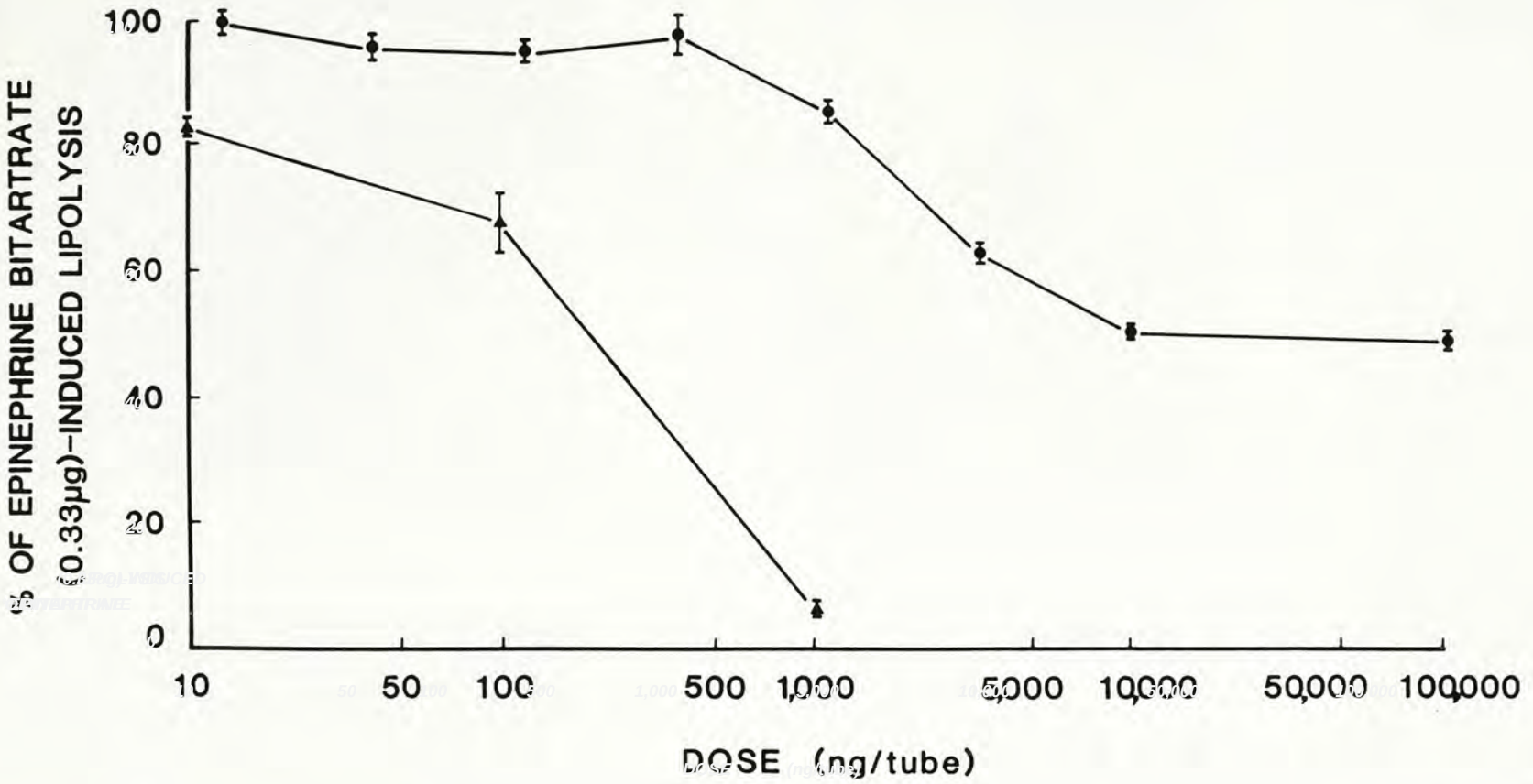


Figure 2-13. Effect of L_5 on epinephrine-induced lipolysis. The inhibition curve of L_5 (●) in the range of 0.5-10 μg was parallel to that of propranolol (▲). Beyond 10 ug, the dose dependency of L_5 lost because of agglutination of adipocytes (see figure 2-14). The inhibition curves of P_{60} and propranolol were shown to be parallel to each other in figure 2-11.

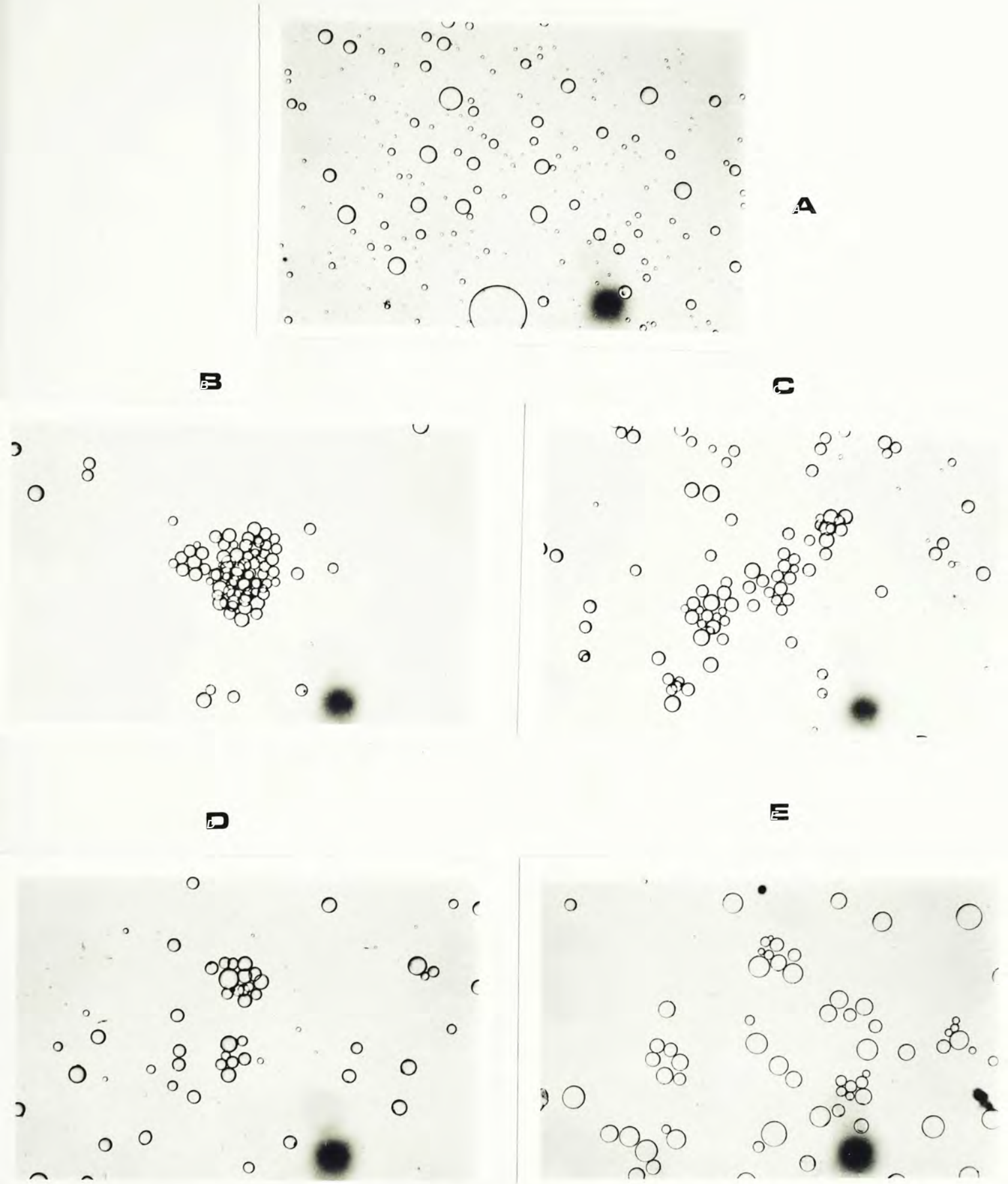


Figure 2-14 Agglutination of rat epididymal fat cells by M. charantia lectin (L_5). Isolated fat cells were incubated with various doses of L_5 for two hours at 37°C before viewing. The photomicrographs show untreated fat cells (A), cells treated with varying dose of L_5 (B-E). B : 500µg/ml, C : 10 µg/ml, D : 3.3 µg/ml and E : 1.1 µg/ml.

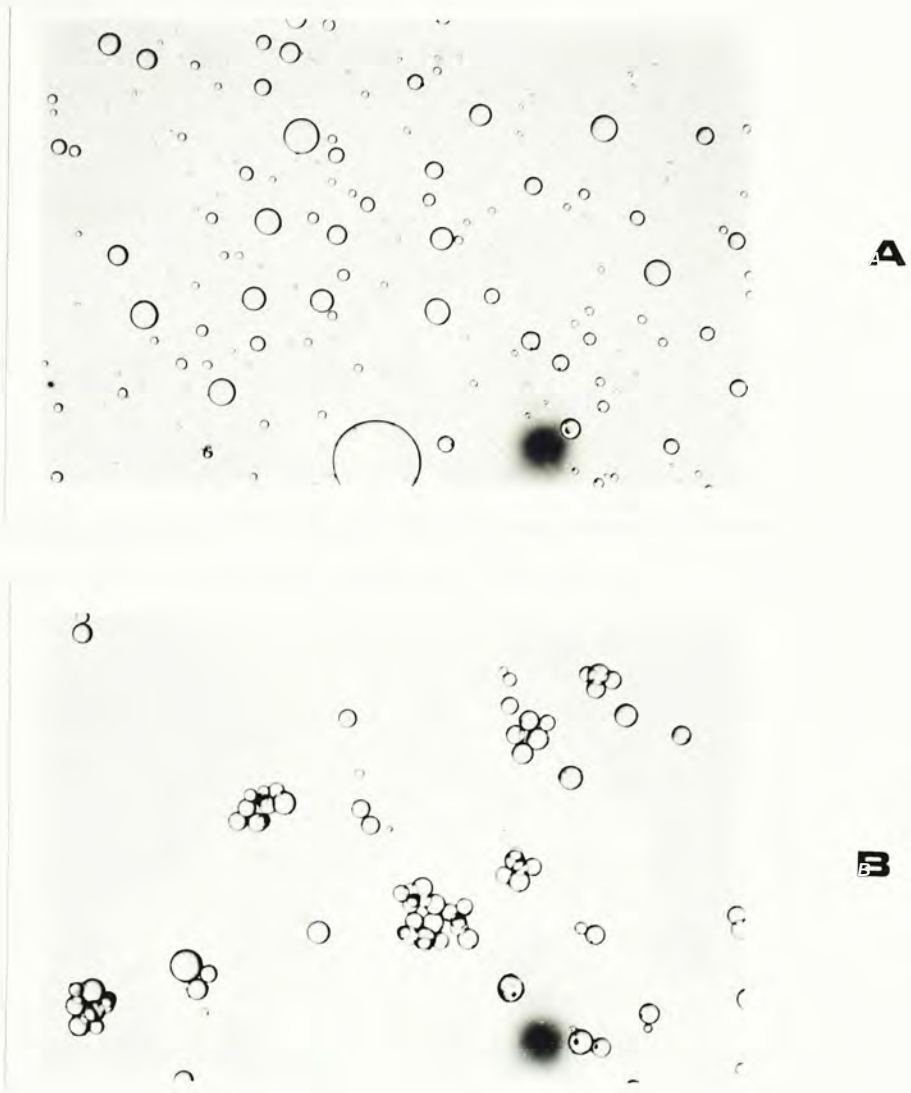


Figure 2-15 Agglutination of rat epididymal fat cells induced by P_{60}. Isolated fat cells were incubated with 500 µg/ml P_{60} for two hours at 37°C before viewing. Photomicrograph(A) shows the untreated fat cells and (B) shows the treated cells which showed considerable agglutination.

Table 2-3

Effects of enzymes, glutathione, galactose and α-methyl-D-mannoside on antilipolytic activities of P_{60} and L_5

Experiment No.	Treatment	% of epinephrine bitartrate (0.33 µg)-induced lipolysis	
		P_{60}	L_5
1	None (control)	61.52 ± 1.38	47.26 ± 1.22
	Trypsin	90.78 ± 0.61[a]	69.49 ± 0.72[a]
	Chymotrypsin	66.11 ± 2.39	73.61 ± 5.09[a]
	Glutathione	117.90 ± 1.45[a]	82.24 ± 0.79[a]
2	None (control)	72.7 ± 1.30	60.10 ± 2.00
	Galactose (25 mM)	101.5 ± 0.40[a]	96.10 ± 0.50[a]
	α-Methyl-D-Mannoside	74.1 ± 1.90	70.30 ± 2.30[b]

The dose of L_5 tested was 50 µg in experiment 1 and 25 µg in experiment 2.

The dose of P_{60} tested was 500 µg in both experiments.

a = $p < 0.001$ compared with control, *b* = $p < 0.5$ when compared with control.

Table 2-4

Assay of L_5 for lipolytic activity

Experiment No.	Fraction assayed	Dose (µg/tube)	Glycerol production (µmol/hr/g fat cell dry wt)
1	None	-	1.60 ± 0.06
	L_5	100	1.53 ± 0.10 (NS)
2	None	-	2.05 ± 0.07
	L_5	2	1.96 ± 0.09 (NS)
		500	1.93 ± 0.03 (NS)

NS = Statistically not significant compared with control.

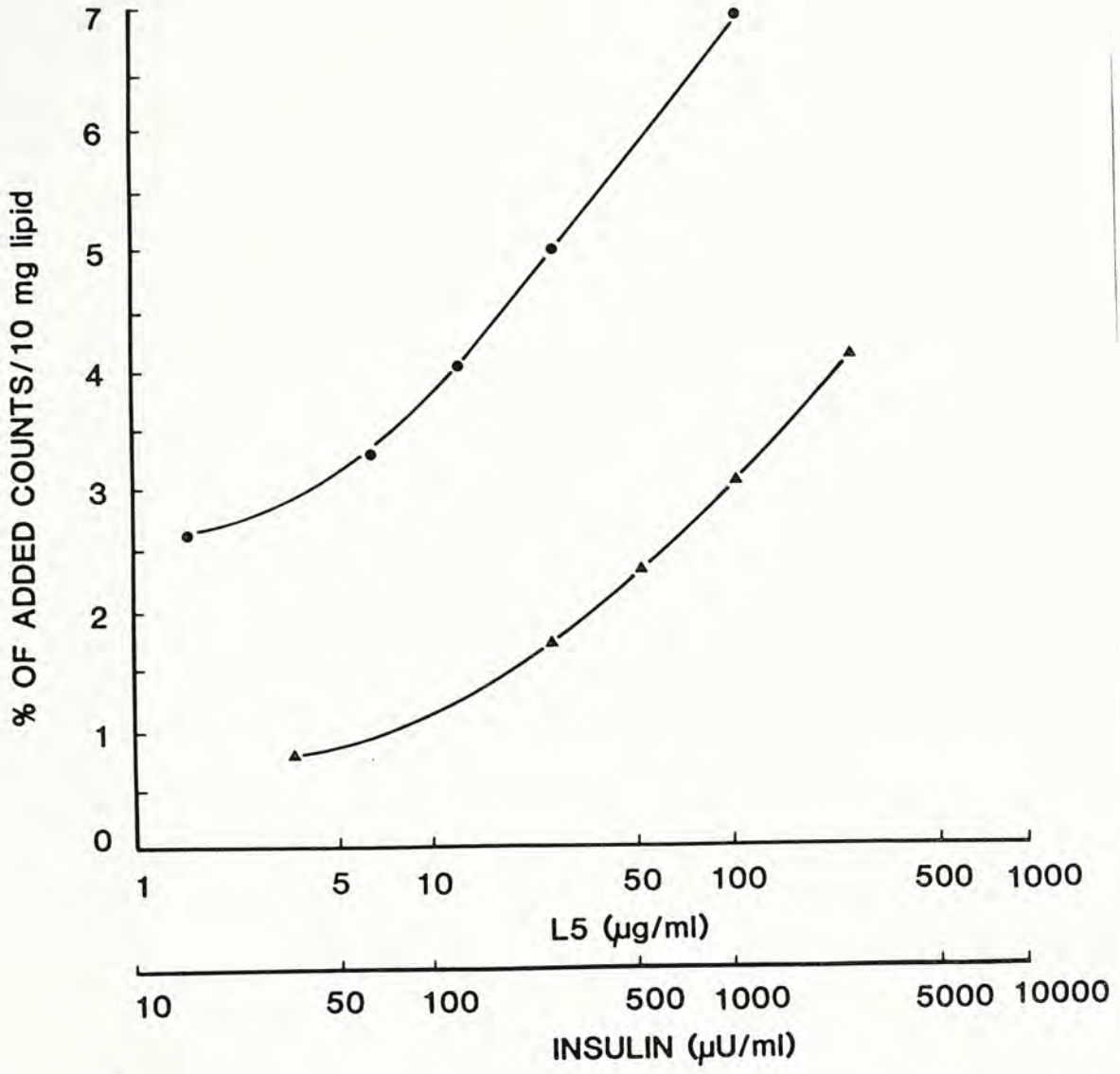


Figure 2-16. Dose response curves of insulin (●) and L_5 (▲) in stimulating the incorporation of [3-3H]-glucose into scintillant extractable total lipids. The result was expressed as percentage of total counts added per 10mg cell total lipids. The parallelism of the two curves suggested that L_5 may act on the insulin receptor or a common effector system with insulin to produce its lipogenic effect.

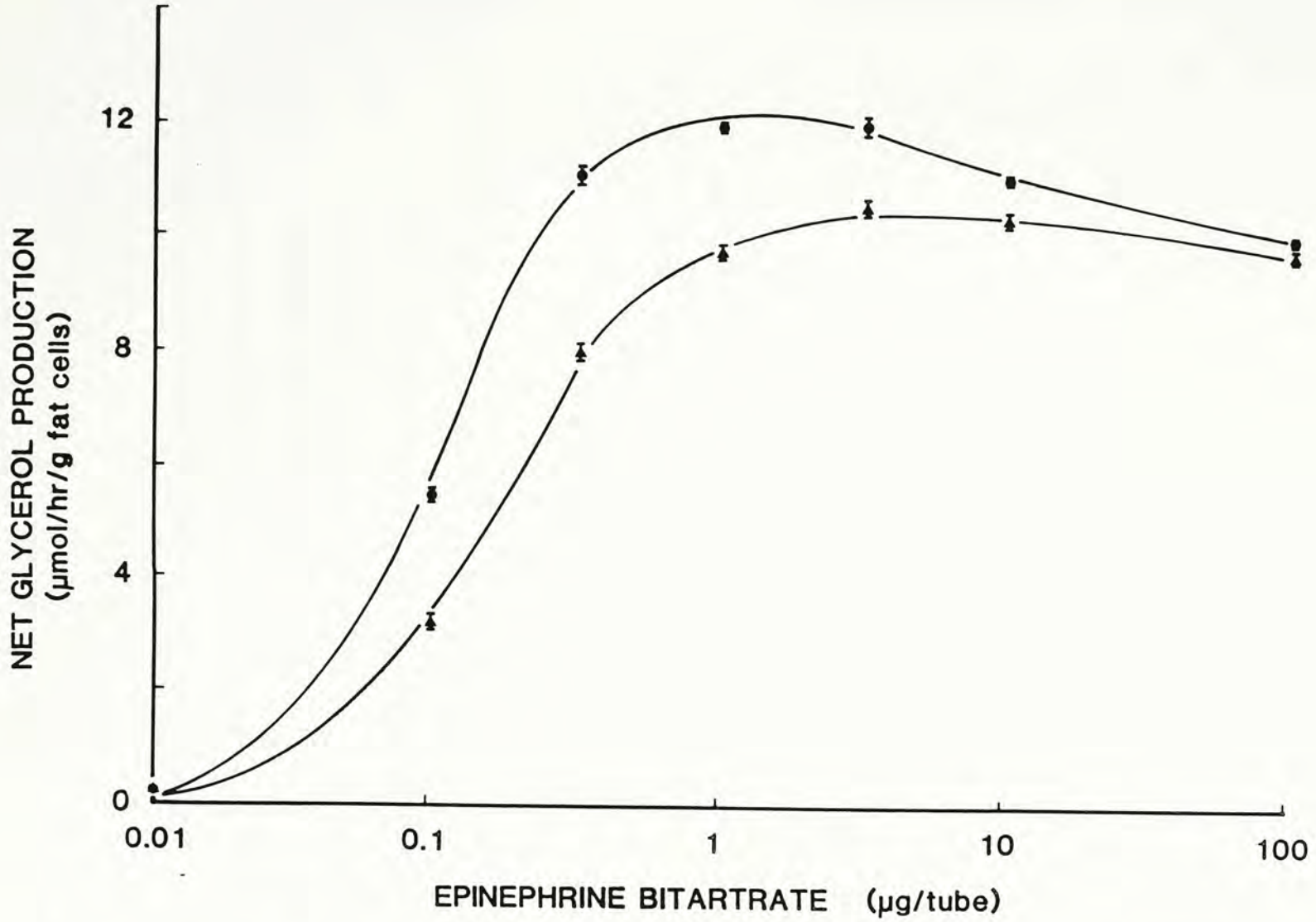


Figure 2-17a. Effect of increasing doses of epinephrine bitartrate (●) on the antilipolytic effect of 500μg P_{60} (▲). The inhibitory effect of P_{60} diminished at high doses (3.3 and 10μg) of epinephrine and finally was overcome by a supramaximal dose (100μg) of epinephrine.

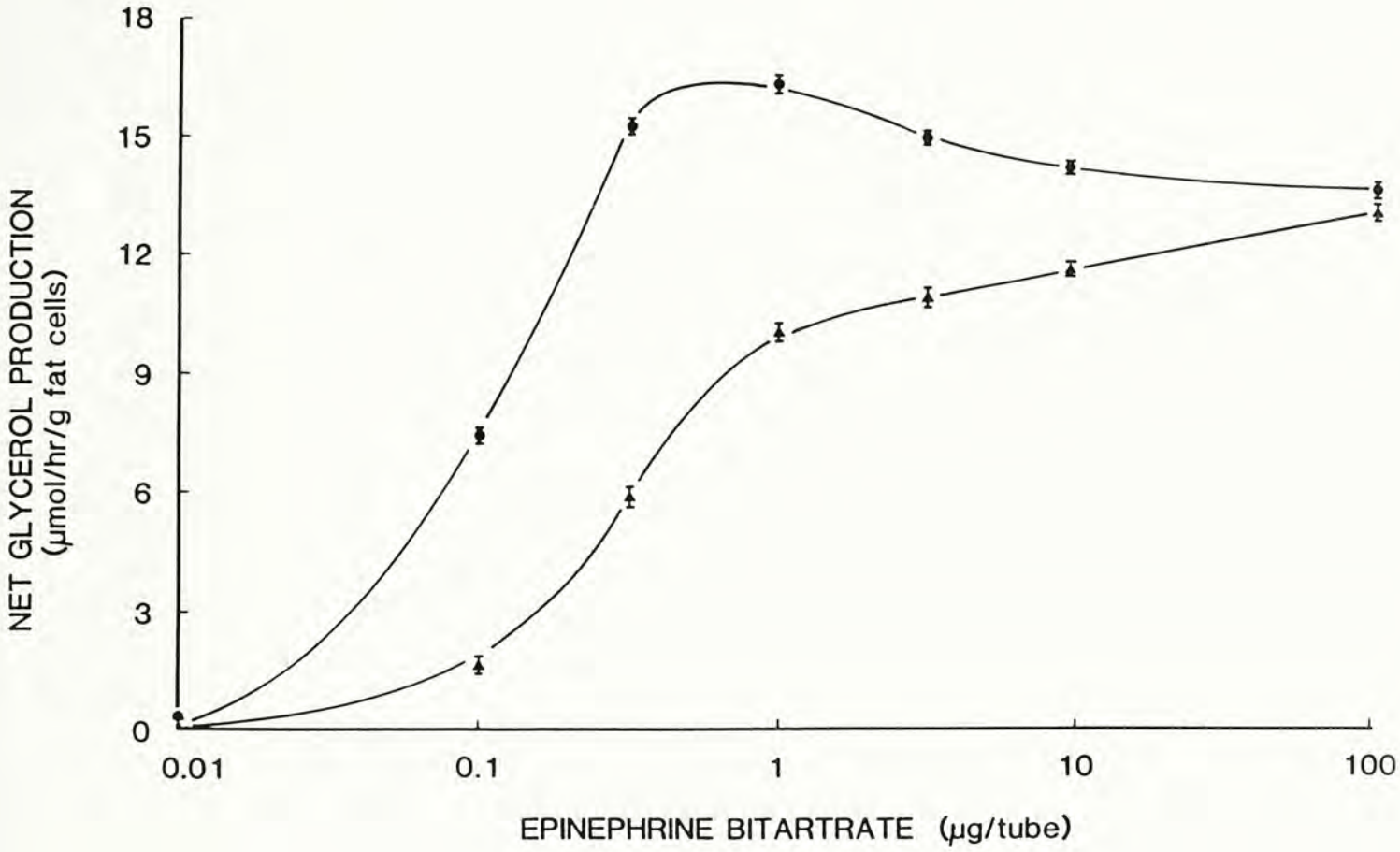


Figure 2-17b. Effect of increasing doses of epinephrine bitartrate (●) on antilipolytic effect of 50μg L_5 (▲). The inhibitory effect diminished like P_{60} at high doses (3.3 and 10μg) of epinephrine and finally was overcome by a supramaximal dose of epinephrine bitartrate. It is noteworthy that the patterns of inhibition of P_{60} and L_5 are very similar.

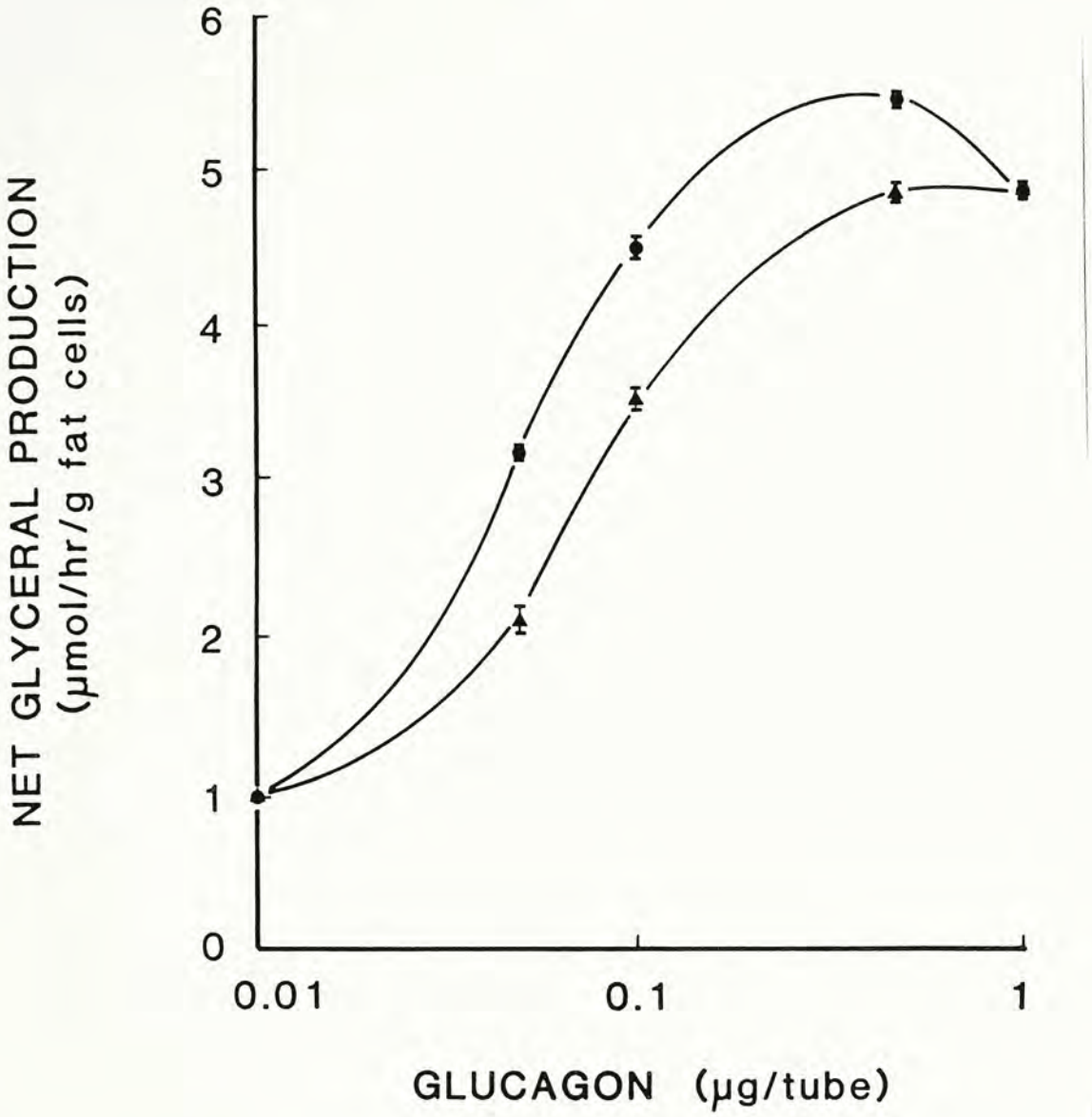


Figure 2-18. Effect of increasing doses of glucagon (●) on anti-lipolytic effect of 50μg L_5 (▲). The inhibitory effect was overcome by a supramaximal dose (1μg) of glucagon.

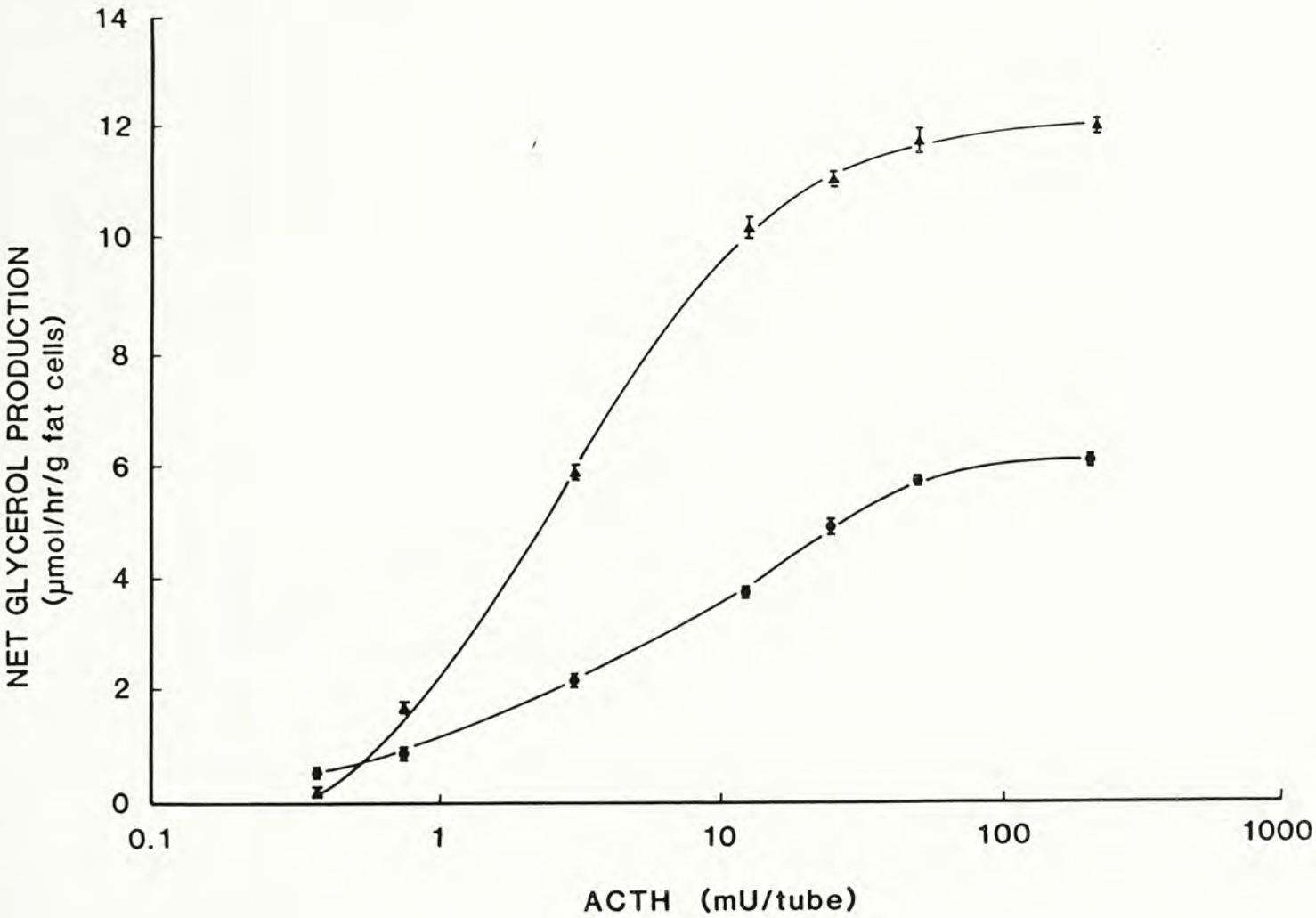


Figure 2-19. Effect of increasing doses of ACTH (▲)on antilipolytic effect of 50μg L_5 (●). The inhibitory effect could not be overcome by raising the concentration of ACTH.

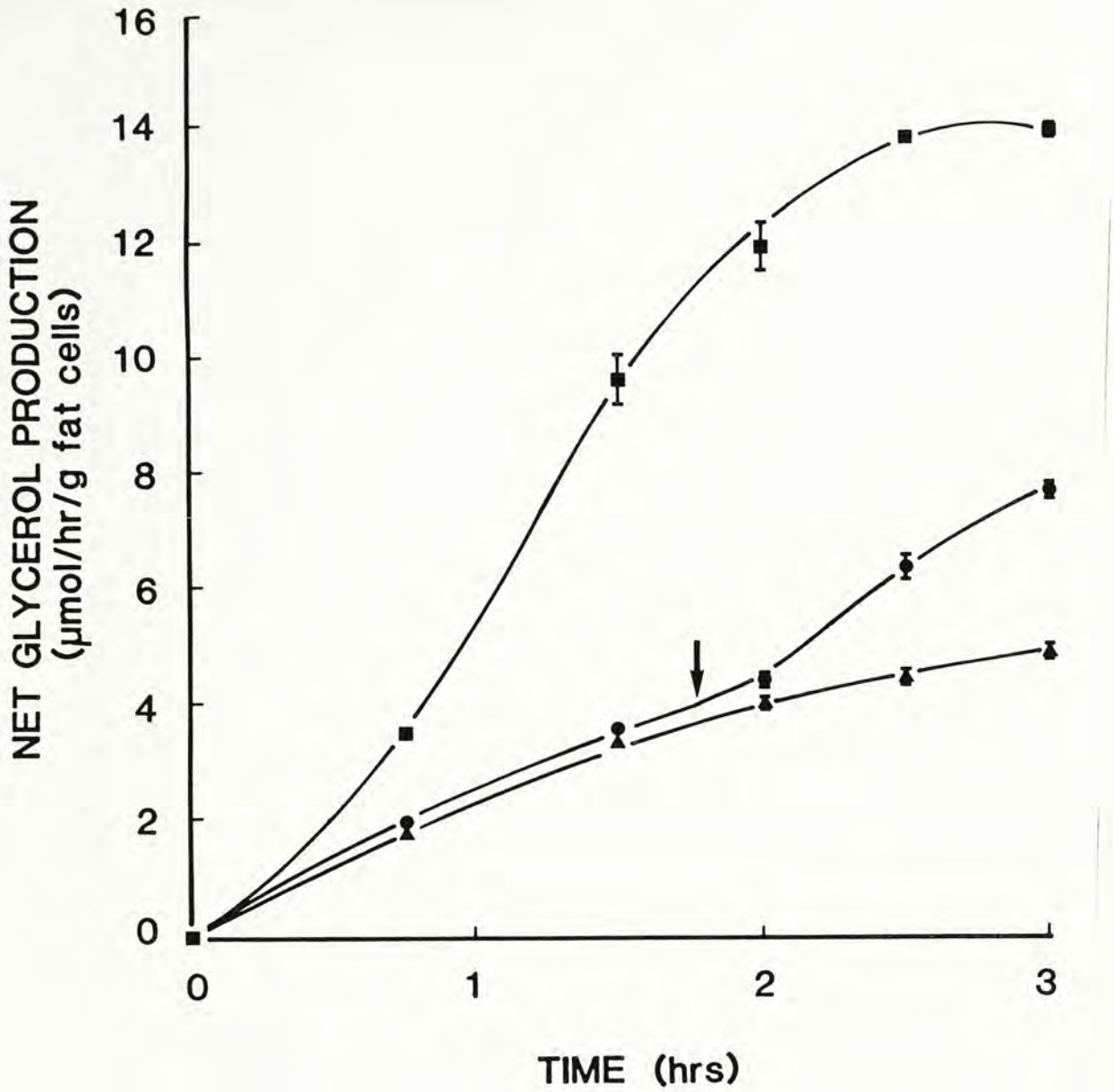


Figure 2-20a. Effect of epinephrine bitartrate (3.3µg) in reversing the inhibitory effect of 2000µg P_{60} on epinephrine bitartrate (0.33µg)-induced lipolysis. Curve (■——■) represents the time course of epinephrine-induced lipolysis. Curve (▲——▲) represents the time course of epinephrine-induced lipolysis in the presence of P_{60}. Curve (●——●) is similar to curve (▲——▲) except that epinephrine bitartrate (3.3µg) was added one hr. 45 min.after the start of the assay, as indicated by the arrow.

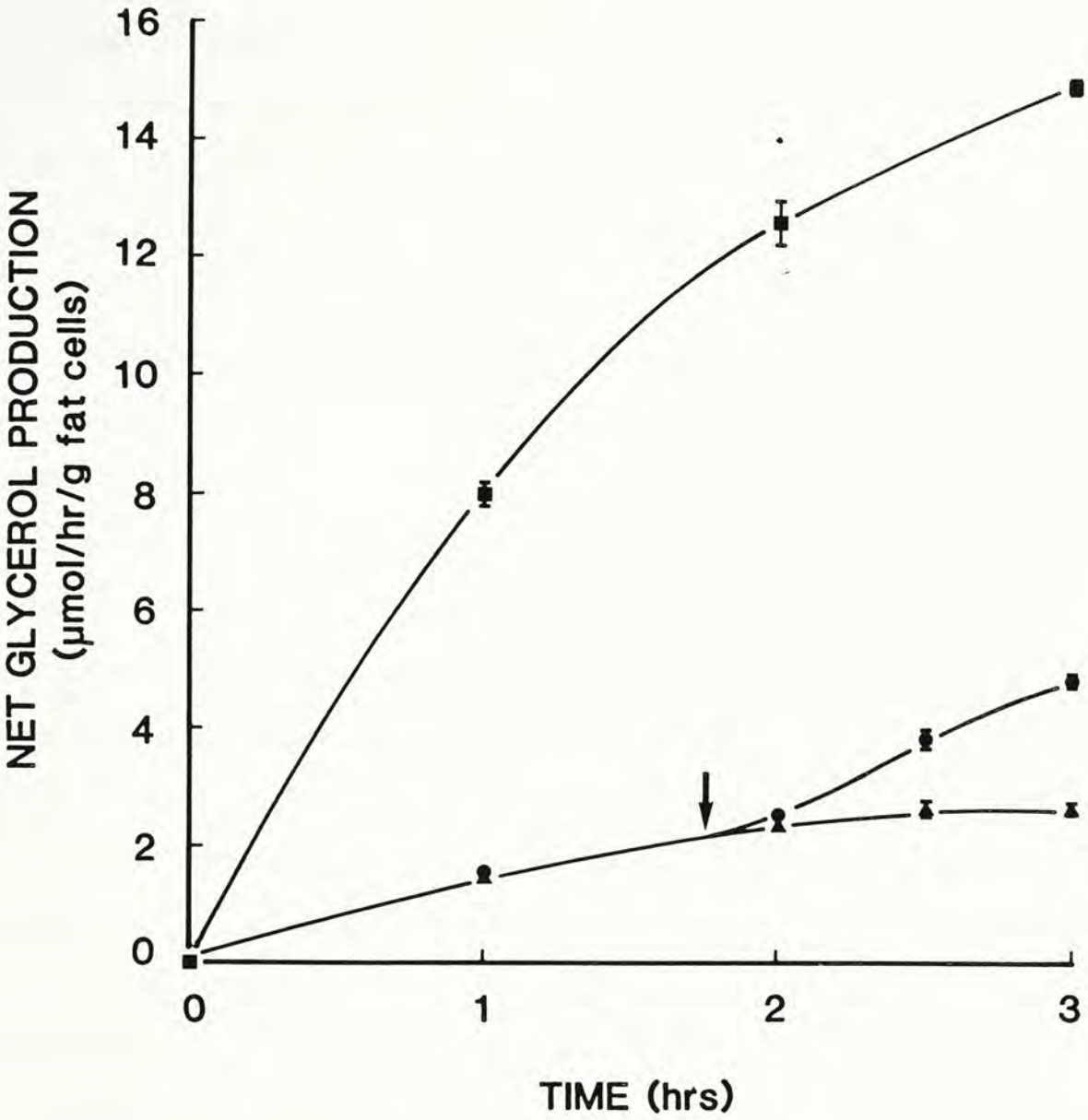


Figure 2-20b. Effect of ACTH (100mU) in reversing the inhibitory effect of 2000µg P_{60} on epinephrine (0.33µg)-induced lipolysis. Curve (■——■) represents the time course of epinephrine-induced lipolysis. Curve (▲——▲) represents the time course of epinephrine-induced lipolysis in the presence of P_{60}. Curve (●——●) is similar to curve (▲——▲) except that ACTH was added one hr. 45 min. after the start of the assay as indicated by the arrow.

before the termination of a usual 2 hour incubation.

The lectin, like P_{60} from which it was derived, had no effect on dibutyryl cyclic AMP-stimulated lipolysis. An acetone powder (AP) of M. charantia seeds yielded similar results (Table 2-5).

Insulin-like activities were detectable in both P_{60}, an intermediate partially purified fraction and the starting material for affinity chromatography on lacto-gel A, and L_5, the final purified lectin preparation. All lines of evidence suggest that most of the cell agglutinating and insulinomimetic activities found in P_{60} coincide and concentrate in L_5. The evidence are enumerated hereinafter. Both P_{60} and L_5 were antilipolytic as well as lipogenic. The antilipolytic activity was heat labile in each case. Furthermore, the antilipolytic activity in P_{60} finding consistent with the protein nature of lectins. The activity was extracted into P_{60}-Ip which gave a lipolysis inhibition curve parallel to those of P_{60} and L_5. L_5 was present as a common electrophoretic band in P_{60} and P_{60}-Ip, but absent from P_{60}-Aq which was devoid of antilipolytic activity. All the electrophoretic bands characteristic of P_{60} were distributed among the fractions (L_1 to L_5) derived from it (data from our laboratory), and thus presumably little or no other antilipolytically active protein(s) was too tenaciously retained on lacto-gel to be eluted by the concentration of galactose used. The antilipolytic activities of P_{60}, and L_5 were similarly sensitive to trypsin and glutathione, in line with the fact that the lectin is a protein with disulfide linkages. The

Table 2-5

Effects of AP, P_{60} and L_5 on dbcAMP-induced lipolysis

Sample	Dose tested (μg/tube)	% of dbcAMP (2 mM)
None	-	100
AP	500	104.0 ± 0.8
	250	108.8 ± 1.0
P_{60}	1000	104.4 ± 0.5
	500	106.9 ± 1.8
L_5	100	100.6 ± 0.16
	10	102.5 ± 0.96

DbcAMP (2 mM) elicited an increase in glycerol production over the control at the rate of 15.65 ± 0.18 μmol glycerol/hr/g fat cell dry wt.

insulinomimetic activities are an intrinsic characteristic of the lectin because galactose but not α-methyl-D-mannoside abolished this characteristic. Such differential sensitivity to galactose and α-methyl-D-mannoside was also found in the antilipolytic activity of P_{60}. Adipocytes aggregated under the influence of appropriate doses of P_{60} and L_5 alike. P_{60} was also similar to L_5 in that it inhibited epinephrine-, corticotropin-, and glucagon- stimulated lipolysis, but was incapable of affecting dibutyryl cyclic AMP-induced lipolysis, and that its inhibition of epinephrine-stimulated lipolysis could be overcome by raising the dose of epinephrine, which could be explained as an evidence for analogous mechanisms of action of both P_{60} and L_5 on the adipocytes.

The lectin was a competitive inhibitor of epinephrine and glucagon in their lipolytic activities in rat adipocytes, but its inhibition of corticotropin-induced lipolysis was of a noncompetitive nature. Its inability to antagonize dibutyryl cyclic AMP-induced lipolysis suggests that the loci of action of the lectin in the adipocyte lie on the plasma membrane. The viability of the adipocytes after interaction with the lectin was demonstrated by the reversal of the antilipolytic activity of a partially purified lectin preparation by the addition of either corticotropin or epinephrine 1 hr 45 min after the start of the interaction. Further evidence of adipocyte viability came from the decreasing efficacy of a constant dose of the lectin to inhibit the lipolytic activity of rising concentrations of either epinephrine or glucagon, and from the lipogenic activity of the lectin.

The antilipolytic activity of the lectin was susceptible to destruction by heat, trypsin, chymotrypsin, glutathione and galactose, indicating that the integrity of the protein moiety, the disulfide linkages, and galactose which is the sugar specifically bound by the lectin, all play an important role in interaction with the adipocyte leading to an expression of this insulin-like activity.

The rat adipocyte lacks functional α-adrenergic receptors (Carpere et al., 1983) and thus epinephrine binds to β-adrenergic receptors only. Our study disclosed that the M. charantia lectin bound to β-adrenergic and glucagon receptors on the plasma membrane of adipocytes and as a consequence inhibited the lipolytic activities of epinephrine and glucagon because the nature of inhibition was competitive. It is interesting to note that the lectin did not bind to the corticotropin receptor although corticotropin-induced lipolysis could be inhibited. The activity of the lectin in inhibiting epinephrine-stimulated lipolysis could be abolished by galactose but not by α-methyl-D-mannoside, indicating that the interaction of the lectin with the β-adrenergic receptor involved the galactose residue. The possibility of an interaction with the insulin receptor awaits investigation.

2.4.3 Saponin fraction

It can be seen from Table 2-6 that low doses of S 60 i.e. 41 ng, 123 ng and 10 µg, potentiated the effect of low doses of corticotropin (3.125 mU) and epinephrine (0.1 µg). A more

Table 2-6

Effect of low doses of S_{60} on lipolysis induced by low dose of corticotropin and epinephrine bitartrate

Fraction assayed	Glycerol production (μmol/hr/g fat cell dry wt)
None	0
ACTH (3.125 mU)	6.14 ± 0.14[a]
ACTH (3.125 mU) + S_{60} (10 μg)	6.88 ± 0.08*
Epinephrine (0.1 μg)	5.11 ± 0.05[b]
Epinephrine (0.1 μg) + S_{60} (10 μg)	5.92 ± 0.19**
Epinephrine (0.1 μg) + S_{60} (123 ng)	5.88 ± 0.03**
Epinephrine (0.1 μg) + S_{60} (41 ng)	6.04 ± 0.01**

* = $p < 0.005$ compared with a

**= $p < 0.005$ compared with b.

thorough study using corticotropin (3.125 mU) and S_{60} (0.04 to 100 µg) yielded similar results (Table 2-7). At higher doses e.g. 250 and 500 µg, S_{60} elicited an inhibitory effect on corticotropin- and epinephrine-stimulated lipolysis (Table 2-7). It is noteworthy that when tested in the range of 40 to 500 µg, S_{60} alone showed a very slight lipolytic activity (Table 2-7).

The antilipolytic activities of S_{60} and the fractions derived from it after n-butanol extraction are presented in Figure 2-21. Most of the activity was extracted into the butanol layer. Some could be recovered from the aqueous layer, and residual activity was present in the interphase. S_{60}-Bu showed an increase in specific activity over S_{60}. The slope of its inhibition curve was steeper than that of propranolol, a β-adrenergic antagonist (Figure 2-21). When glucagon was used instead of epinephrine to provide the lipolytic stimulus, essentially similar results were obtained with S_{60} and S_{90} (Table 2-8). The antilipolytic activities present in S_{60}, S_{90}, and their derived fractions, were retained after heat treatment (Table 2-9). S_{60}, S_{90} and S_{95} were all capable of inhibiting lipolysis induced by either dbcAMP alone or by dbcAMP and theophylline together (Table 2-10).

Increasing the dose of corticotropin was ineffective in reversing the antilipolytic effect of 50 µg propranolol (Figure 2-22a). A maximal dose of epinephrine bitartrate reversed the antilipolytic effect of 0.05 µg propranolol (Figure 2-22b).

The inhibition of epinephrine-, corticotropin-, and glucagon-induced lipolysis by S_{60} could not be overcome by increasing the dose of the respective lipolytic hormone (Figure

Table 2-7

Lipolytic activity of S_{60} and effect of S_{60} on lipolysis induced by low doses of corticotropin and epinephrine bitartrate

Dose of S_{60} (μg/tube)	Glycerol production (μmol/hr/g fat cell dry wt)		
	S_{60} alone	S_{60} + 3.125 mU ACTH	S_{60} + 0.1 μg epinephrine bitartrate
0	ND	9.32 ± 0.17 *	6.31 ± 0.05 *
0.04	ND	10.38 ± 0.28 c	-
0.12	ND	10.65 ± 0.41 b	-
0.37	ND	9.80 ± 0.04 b	-
10	ND	10.25 ± 0.38 a	-
20	ND	10.91 ± 0.27 d	-
40	0.01 ± 0.005	10.68 ± 0.58 a	-
60	0.053± 0.007	11.95 ± 0.43 a	6.76 ± 0.28 (NS)
100	0.14 ± 0.03	13.18 ± 0.59 a	6.99 ± 0.37 (NS)
250	0.12 ± 0.03	6.92 ± 0.274 d	3.23 ± 0.39 d
500	0.36 ± 0.02	0.54 ± 0.01 d	0.52 ± 0.04 d

ND = undetectable, NS = no statistically significant difference from a = $p < 0.05$, b = $p < 0.0025$, c = $p < 0.01$, d = $p < 0.005$, when compared with *

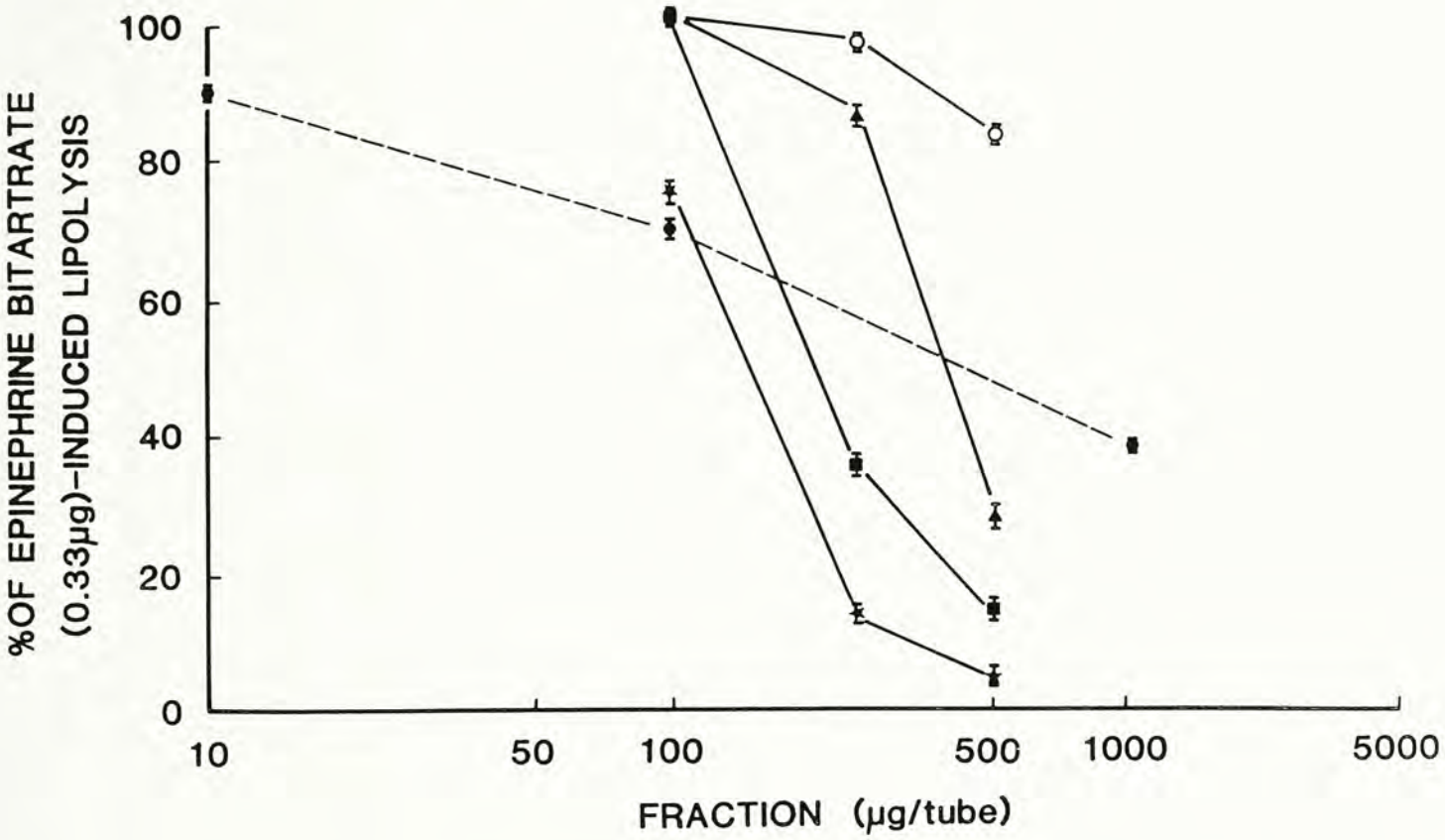


Figure 2-21 Effect of fractions derived from S_{60} after n-butanol extraction on epinephrine-induced lipolysis. Most of the antilipolytic activity of S_{60} (■) was extracted into S_{60}-Bu (★). Some was present in S_{60}-Aq(▲) and only very little could be recovered from S_{60}-Ip (○). The slope of inhihition curve of propranolol (●----●) was less steep than that of S_{60} and S_{60}-Bu, implying that the sites of their antilipolytic action might be different.

Table 2-8

Effect of S_{60} and S_{90}, and fractions derived from them after n-butanol extraction on glucagon-induced lipolysis

Dose (µg/tube)	% of glucagon (0.2 µg)-induced lipolysis					
	S_{60}	S_{60}-Aq	S_{60}-Bu	S_{90}	S_{90}-Aq	S_{90}-Bu
500	15.8 ± 1.7	56.0 ± 0.6	0	12.1 ± 1.8	18.6 ± 0.3	13.5 ± 1.7
250	58.3 ± 4	101.8 ± 2.4	0	87.3 ± 1.5	77.2 ± 3.4	10.4 ± 0.7
100	150.3 ± 4.5 ^{c}	107.5 ± 0.2 ^{b}	65.9 ± 3.2	117.6 ± 4.5 ^{b}	130.4 ± 4.2 ^{c}	105.7 ± 1.8 ^{a}

Glucagon (0.2 µg) elicited an increase of glycerol production over the control at a rate of 3.0 ± 0.06 µmol/hr/g fat cell dry wt.

a = $p < 0.05$, b = $p < 0.01$ and c = $p < 0.001$, compared with glucagon-induced lipolysis which was taken as 100%

S_{90} was the supernatant obtained after adding $(NH_4)_2SO_4$ to a saline extract of *M. charantia* seeds to 90% saturation.

Table 2-9

Effect of heat treatment on antilipolytic activities of S_{60}, S_{90} and fractions derived from them after n-butanol extraction

Fraction assayed	Dose (μg/tube)	% of ACTH (25 mU)-induced lipolysis		% of epinephrine bitartrate (0.33 μg)-induced lipolysis	
		No heat treatment	Heat-treated	No heat treatment	Heat-treated
S_{60}	500			11.2 ± 0.9	4.9 ± 0.5
	250	-	-	33.3 ± 0.7	33.8 ± 2.9
	100			100.8 ± 2.5	92.5 ± 0.8
S_{60}-Bu	250			5.5 ± 0.9	11.9 ± 0.2
	100	-	-	40.8 ± 0.5	51.1 ± 1.7
	50			91.2 ± 1.9	93.5 ± 2.2
S_{60}-Aq	500	-	-	71.8 ± 0.5	76.4 ± 1.4
S_{60}-Ip	500	-	-	95.3 ± 1.2	89.1 ± 1.4
S_{90}	500			1.1 ± 0.2	-
	250	-	-	16.1 ± 0.5	-
	100			85.3 ± 5.3	-
S_{90}-Bu	500	3.1 ± 0.8	2.6 ± 0.3	0.8 ± 0.4	0.8 ± 0.3
	250	0.7 ± 0.1	2.1 ± 0.6	1.6 ± 1.3	1.5 ± 0.9
	100	38.4 ± 2.4	64.9 ± 3.3	31.5 ± 1.7	38.1 ± 2.9
	50	98.2 ± 2	124.4 ± 5.6	98.8 ± 1.9	86.0 ± 5.6
S_{90}-Aq	500	1.3 ± 0.4	1.8 ± 0.5	0.44± 0.4	1.4 ± 1.0
	250	42.1 ± 5.0	52.2 ± 2.4	26.7 ± 1.4	25.8 ± 2.3
	100	85.2 ± 3.1	112.7 ± 2.6	79.2 ± 4	80.0 ± 1.4
	50	95.6 ± 4.3	111.1 ± 1.1	100.8 ± 6.1	80.2 ± 5.2

ACTH (25 mU) and epinephrine bitartrate (0.33μg) elicited an increase of glycerol production at a rate of 11.78 and 4.21 umol glycerol/hr/g fat cell dry wt.

Table 2-10

Effect of S_{60}, S_{90} and S_{95} on lipolysis induced by dbcAMP or co-induced by dbcAMP and theophylline.

Fraction assayed	Dose (µg/tube)	% of dbc AMP (2 mM) -induced lipolysis	% of dbc AMP (2 mM) and theophylline (1 mM)-coinduced lipolysis
S_{60}	500	18.3 ± 1.0	10.4 ± 0.2
	250	42.3 ± 1.1	10.3 ± 0.2
S_{90}	500	4.0 ± 0	4.2 ± 0.3
	250	28.5 ± 0.8	11.9 ± 0.5
S_{95}	500	9.1 ± 0.4	5.4 ± 0.1
	250	39.6 ± 2.1	18.9 ± 1.7

DbcAMP (2 mM) elicited an increase of glycerol production over the control at a rate of 15.65 ± 0.18 µmol glycerol/hr/g fat cell dry wt. The corresponding value for dbc AMP (2 mM) and theophylline (1 mM)-coinduced lipolysis was 9.41 ± 0.15 in another experiment.

S_{90} and S_{95} were the supernatants obtained after adding $(NH_4)_2SO_4$ to a saline extract of *M. charantia* seeds to 90% and 95% saturation respectively.

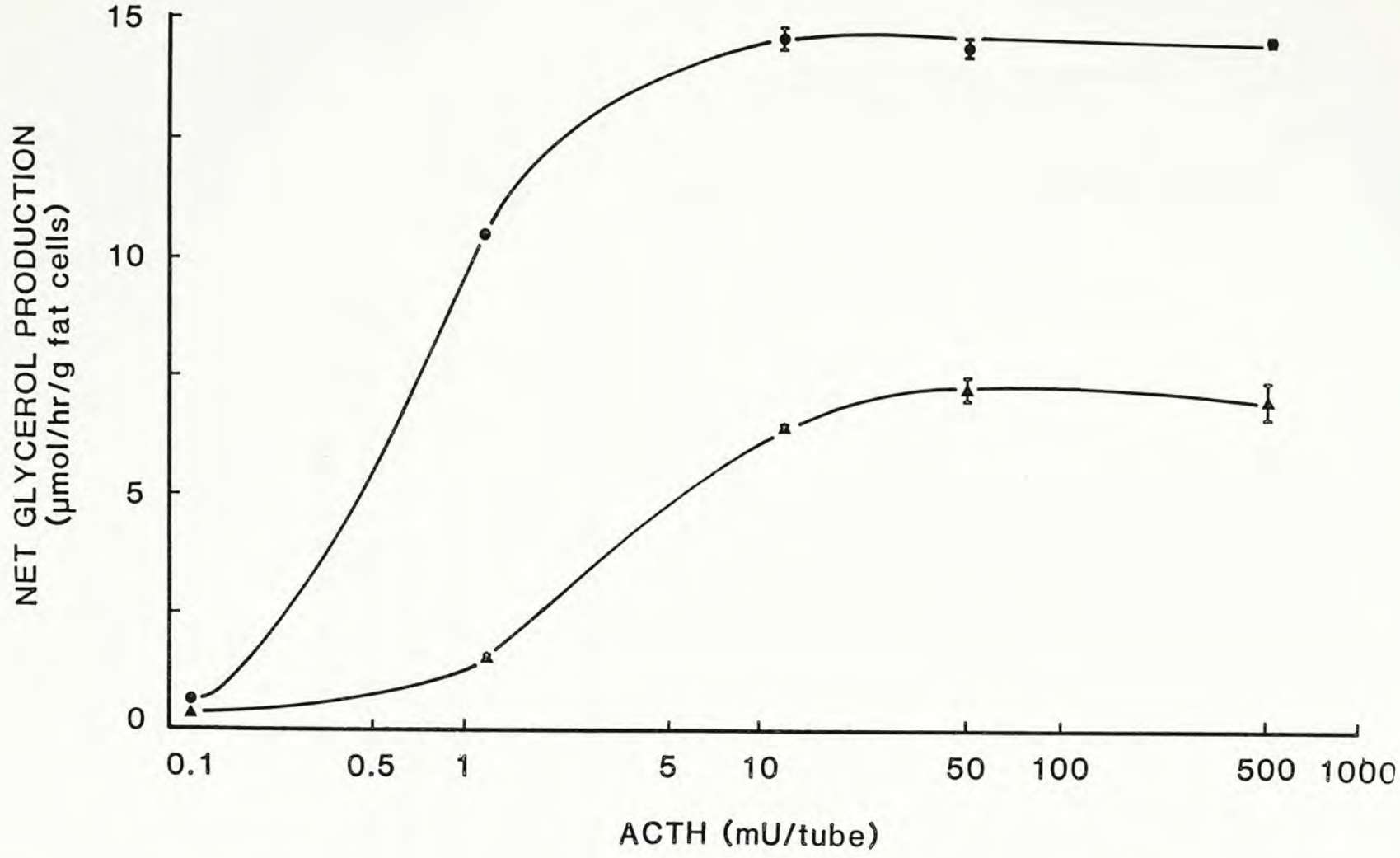


Figure 2-22a. Effect of propranolol (100 μg) on lipolysis induced by varying dose of corticotropin (●). The inhibitory effect of propranolol (▲)could not be overcome by increasing the dose of ACTH.

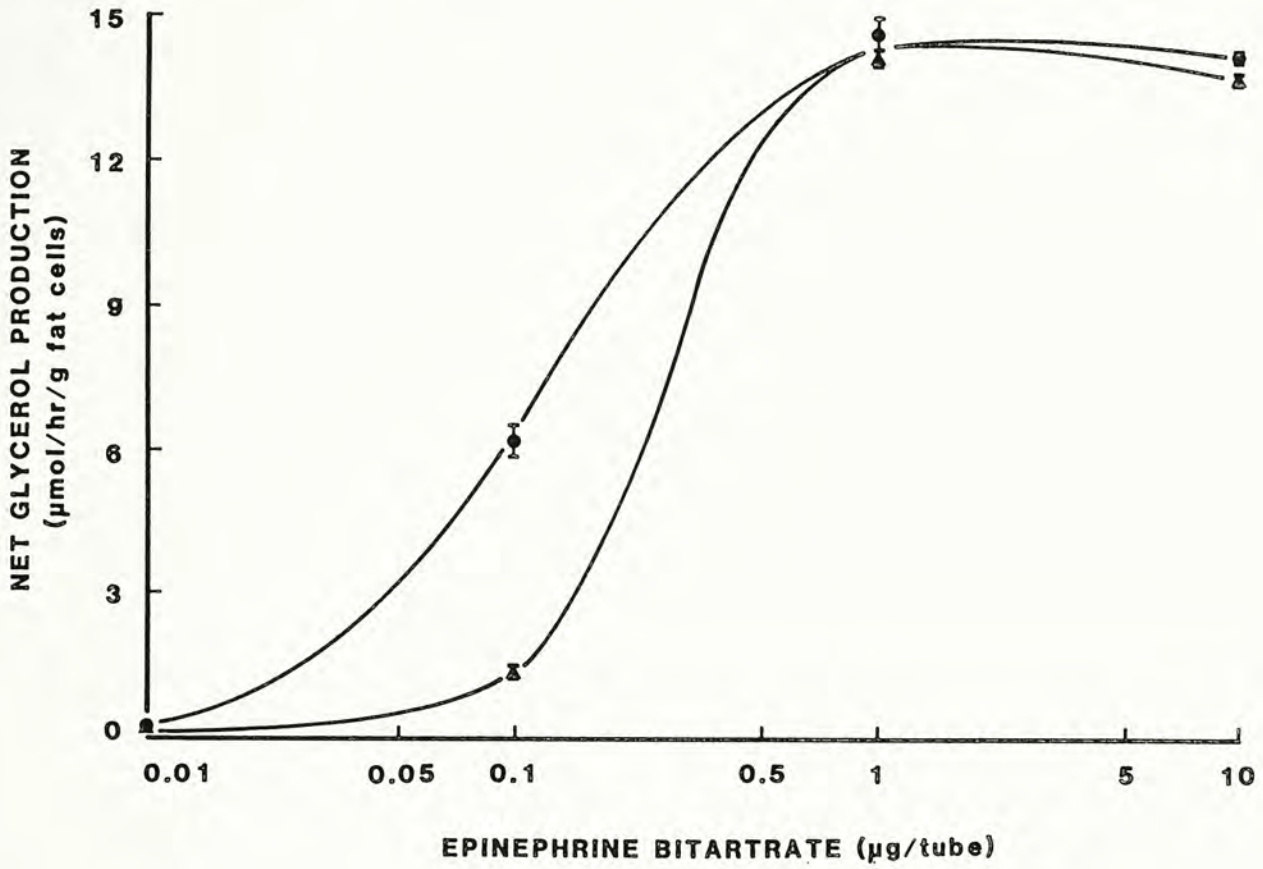


Figure 2-22b. Effect of increasing doses of epinephrine bitartrate (●) on antilipolytic effect of 0.05 µg propranolol (▲). The inhibitory effect was overcome by a maximal dose (1 µg) of epinephrine bitartrate.

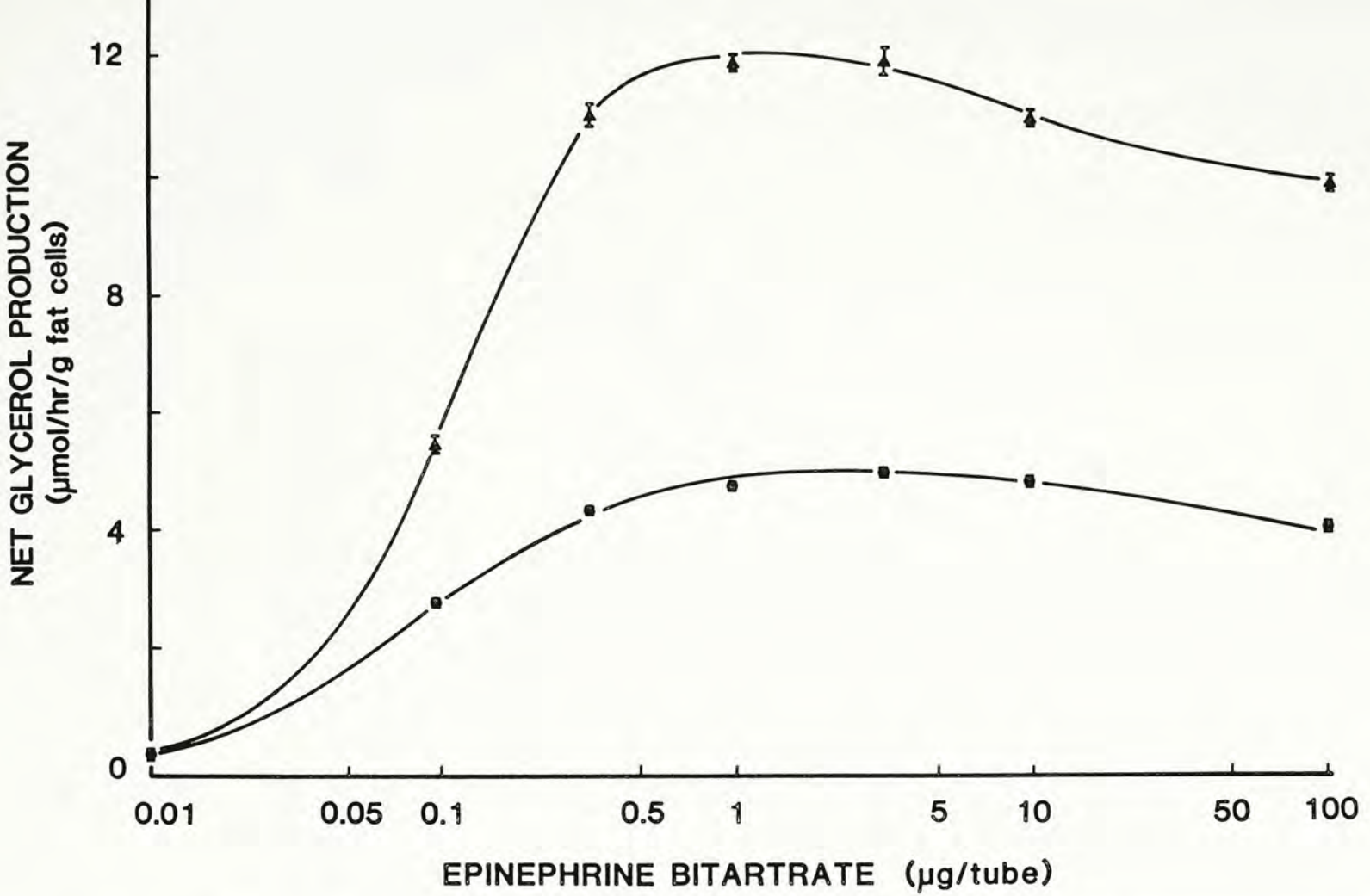


Figure 2-23. Effect of increasing doses of epinephrine bitartrate (▲)on antilipolytic effect of 250 µg S_{60} (●). The inhibitory effect of S_{60} could not be overcome by increasing the dose of the hormone.

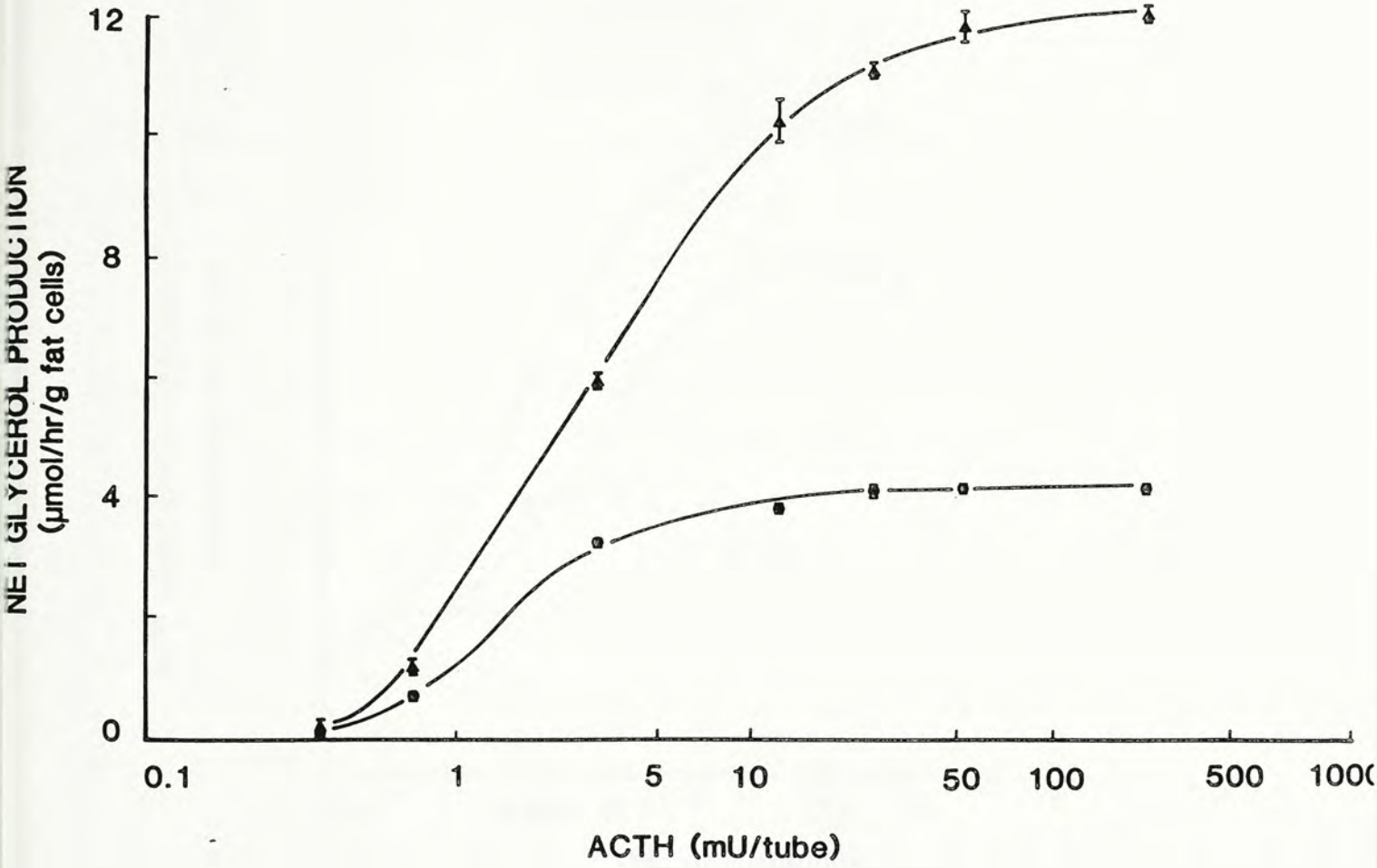


Figure 2-24. Effect of increasing doses of ACTH (▲) on antilipolytic effect of 250 µg S_{60} (●). The inhibitory effect of S_{60} could not be overcome by increasing the dose of the hormone.

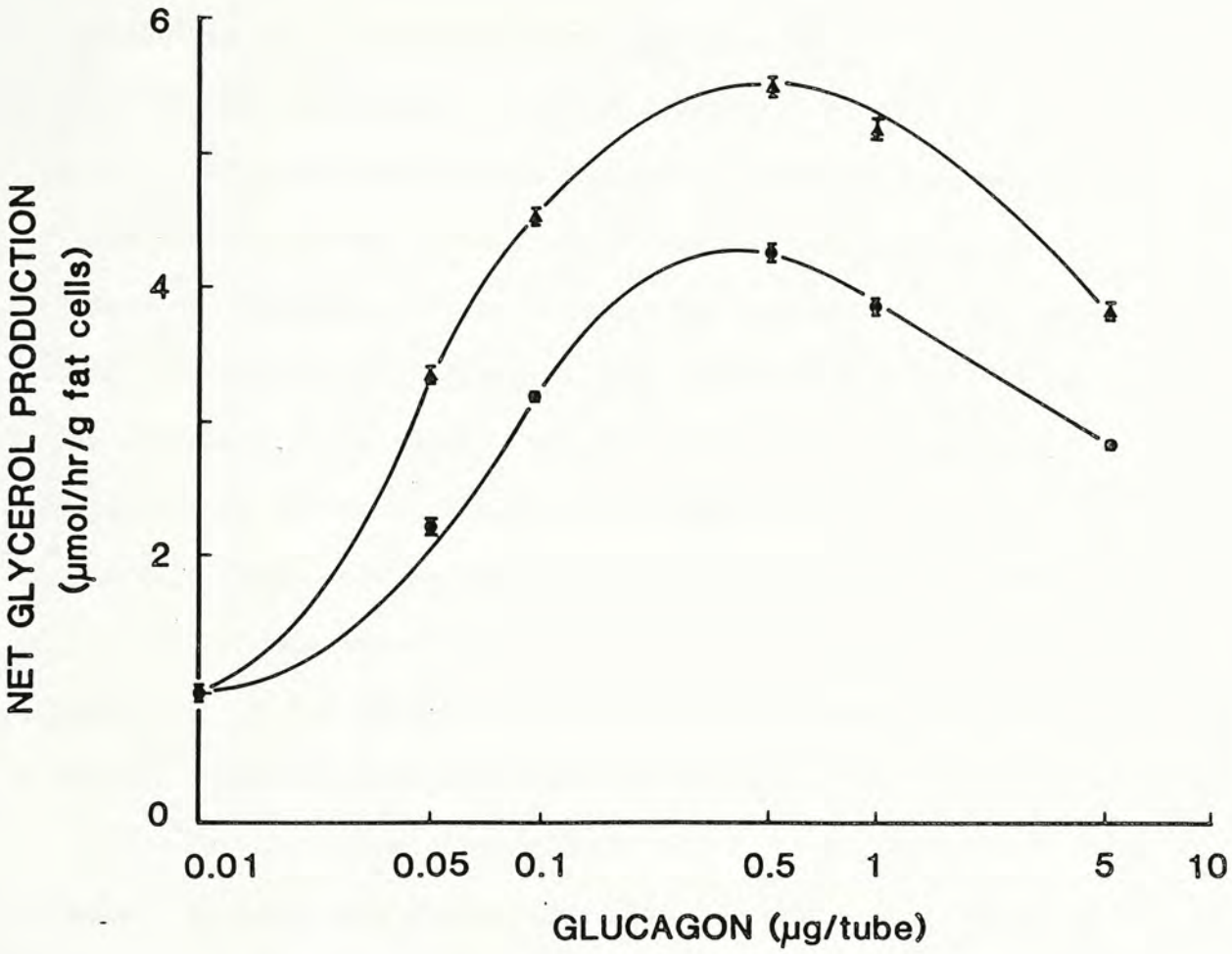


Figure 2-25. Effect of increasing dose of glucagon (▲)on antilipolytic effect of 250 μg S_{60} (●). The inhibitory effect of S_{60} could not be overcome by increasing the dose of the hormone.

2-23, 24, and 25).

The inhibitory effect of propranolol on epinephrine-induced lipolysis could be partially reversed at various time intervals up to 1 hr 45 min after the start of the assay, by adding a submaximal dose (25 mU) of corticotropin (Figure 2-26). However, a similar assay system applied to test the reversibility of the antilipolytic effect of S_{60} showed that 500 µg S_{60} produced an inhibition which could not be reversed by addition of either 25 mU corticotropin or 3.3 µg epinephrine 1 hr 45 min after the start of the assay (Figure 2-27). When the dose of S_{60} was lowered to 250 µg in a subsequent experiment, its antilipolytic effect could only be very slightly reversed by the addition of, 1 hr 45 min after the start of the assay, either 3.3 µg epinephrine or 100 mU corticotropin which are doses that produce a maximal lipolytic response (Figure 2-28).

The antilipolytic effect of S_{60} was resistiant to trypsin, emulsin and glutathione (Table 2-11). S_{60} tested at dose of 250 and 500 µg exhibited a marked inhibition of incorporation of D-(3-3H)-glucose into lipid (Table 2-12). The light microscopic appearance of adipocytes at the end of a 2 hr incubation with 500 µg S_{60} was normal. There was neither aggregation nor lysis of the cells (Figure 2-29).

S_{60}-Bu is a purified saponin fraction prepared from M. charantia seeds. Its saponin nature was further substantiated by its heat stability and by its resistance to trypsin and glutathione.

Of the three fractions derived from S_{60} after n-butanol

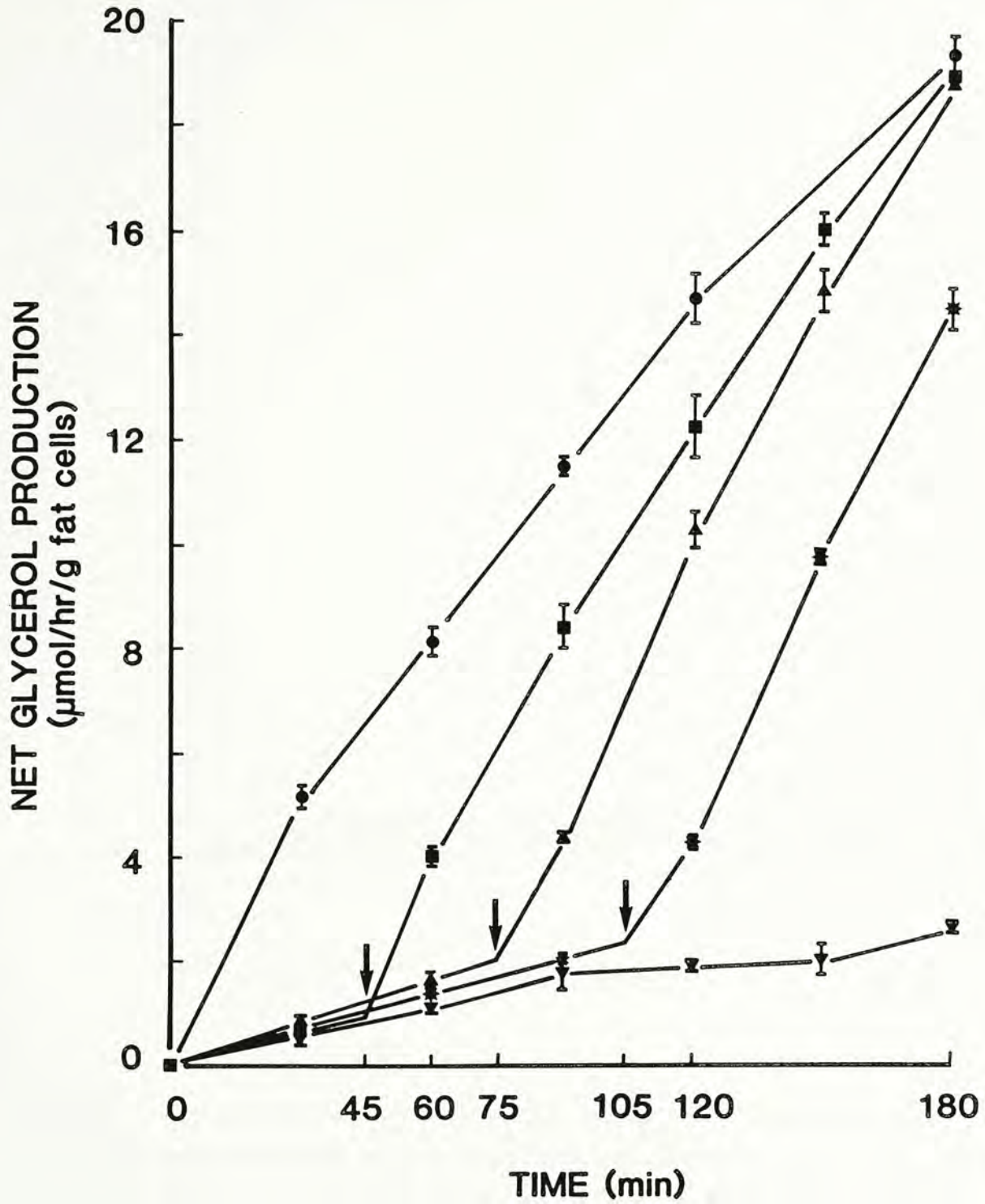


Figure 2-26. Reversal of inhibitory effect of propranolol on epinephrine-induced lipolysis by ACTH. Glycerol release induced by 0.33 μg epinephrine bitartrate (●) was greatly suppressed by 0.5 μg propranolol (▼). The inhibitory effect of propranolol was reversed by adding a submaximal dose (25 mU) of ACTH (indicated by the arrows) at 45 min. (■), 75 min. (▲) and 105 min. (✱) after the start of the assay.

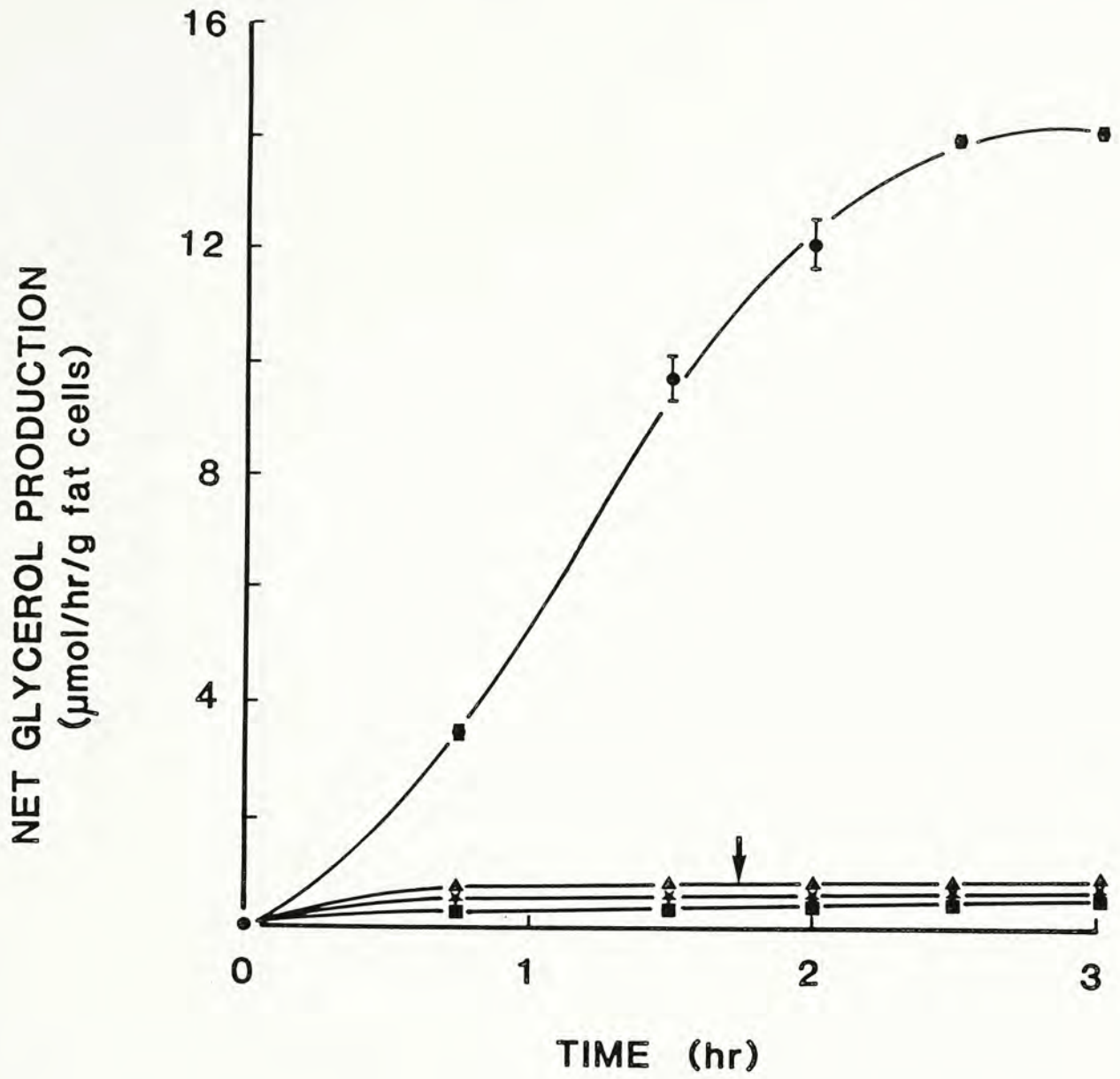


Figure 2-27. Effect of high doses of ACTH and epinephrine on the reversibility of the antilipolytic effect of S_{60}. Glycerol production induced by 0.33 μg epinephrine bitartrate (●) was greatly suppressed by 500 μg S_{60} (■). The inhibitory effect of S_{60} could not be reversed by addition of either 25 mU ACTH (▲) or 3.3 μg epinephrine bitartrate (★) (indicated by the arrow) one hr. 45 min. after the start of the assay.

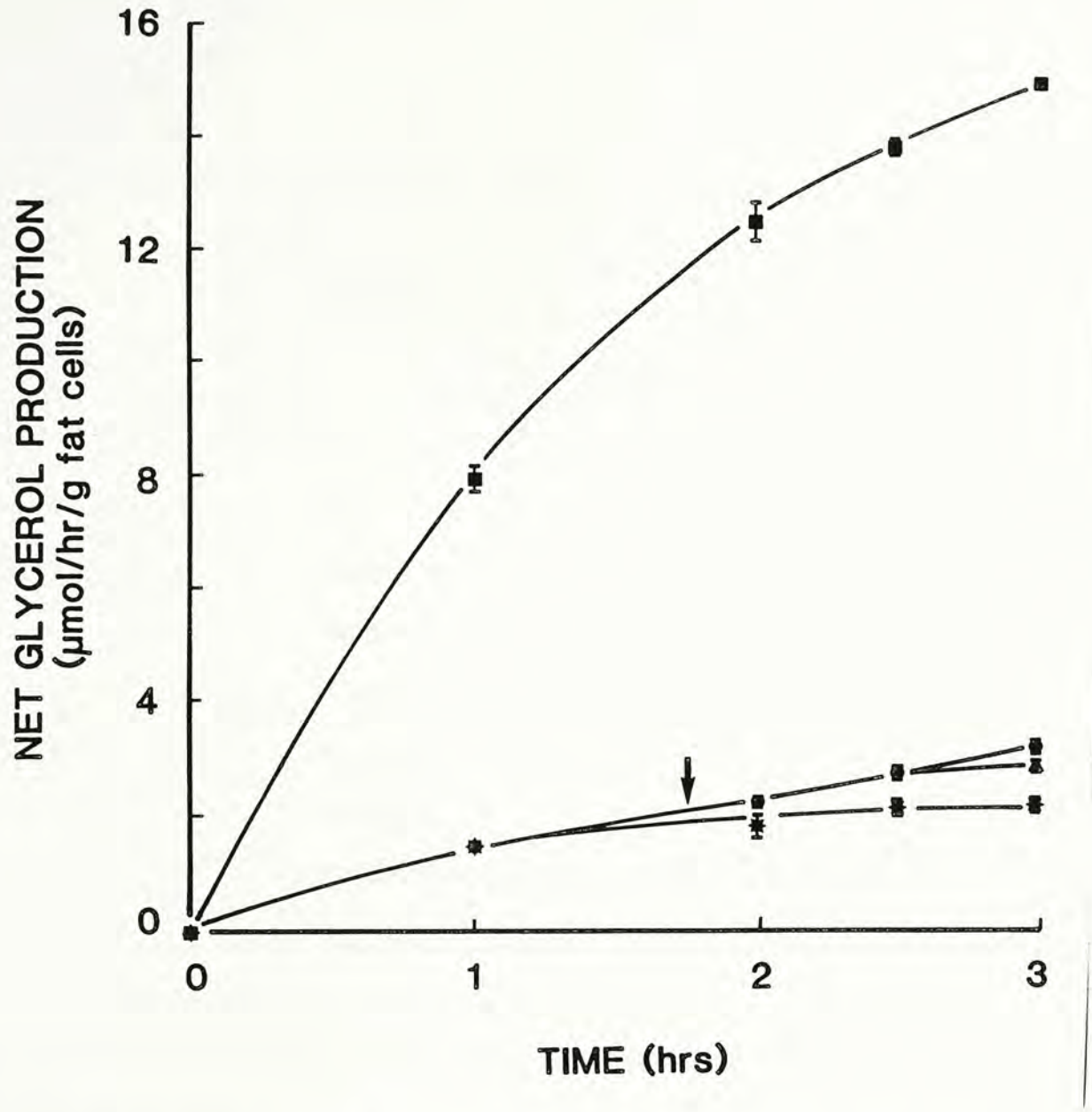


Figure 2-28. Effect of ACTH (100 mU) and epinephrine bitartrate (3.3 µg) in reversing the inhibitory effect of 250 µg S_{60} on epinephrine (0.33 µg)-induced lipolysis. Curve (■——■) represents the time course of epinephrine bitartrate-induced lipolysis. Curve (✸——✸) represents the time course of epinephrine-induced lipolysis in the presence of S_{60}. Curve (●——●) and (▲——▲) are similar to curve (✸——✸) except that ACTH and epinephrine bitartrate were added respectively one hr. 45 min. after the start of the assay, as indicated by the arrow.

Table 2-11

Effect of enzymes and glutathione on antilipolytic activity of S_{60}.

	Treatment of S_{60}	Glycerol production (μmol per hr per g fat cell dry wt)
Buffer	N.A.	0
Epinephrine	N.A.	15.17 ± 0.25
Epinephrine+S_{60}	None	2.81 ± 0.02
	Trypsin	2.85 ± 0.02
	Chymotrypsin	2.91 ± 0.04
	Pronase	3.06 ± 0.06
	Emulsin	2.94 ± 0.05
	Glutathione (2 mM)	3.04 ± 0.01

N.A. = Not applicable

The doses of epinephrine and S_{60} were respectively 0.33 μg and 500 μg throughout.

Enzymes were added at an enzyme:substrate ratio of 1:50 (w/w).

Table 2-12

Antilipogenic effect of S_{60}.

Fraction assayed	Dose (µg/vial)	D-[3-3H]-glucose incorporated into lipid (cpm)
None	-	3059 ± 154
S_{60}	100	2318 ± 246 ($p < 0.05$)
	250	316 ± 7 ($p < 0.001$)
	500	282 ± 18 ($p < 0.001$)

D-[3-3H-]Glucose (90,000 cpm) and about 2×10^4 adipocytes were added to each assay vial.

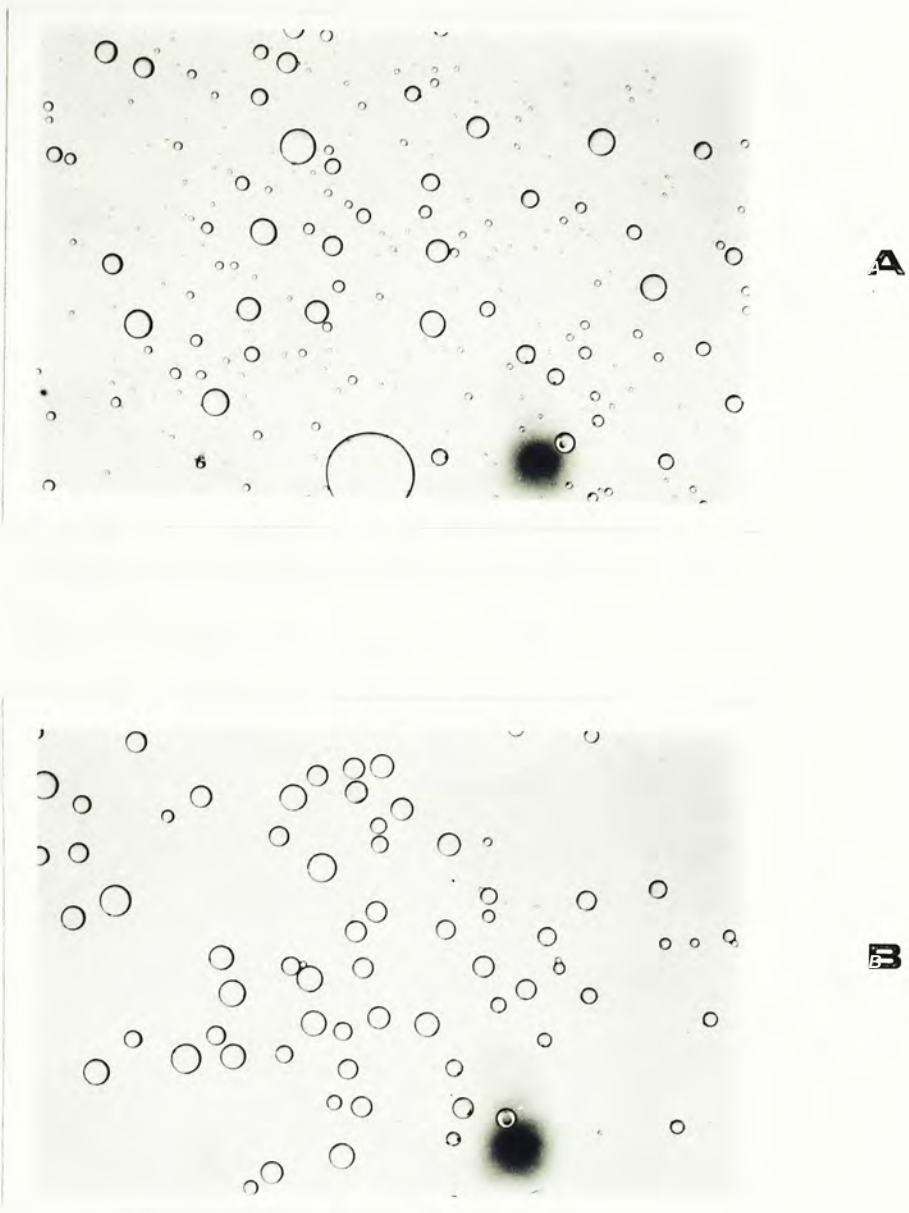


Figure 2-29 Effect of S_{60} on the morphology of isolated fat cells. Isolated fat cells were incubated with 500 µg/ml S_{60} for two hours at 37°C before viewing. There was no observable changes in the morphology of treated cells(B)compared with untreated cells (A).

extraction, S_{60}-Bu showed the highest antilipolytic activity. Its higher specific activity than that of S_{60} was due to the removal of protein contaminants which were extracted into the aqueous layer. It was found that the antilipolytic activity characteristic of S_{60} could also be recovered from the supernatant, after addition to a saline extract of the M. charantia seeds, of ammonium sulfate to 90% or 95% instead of 60% saturation. S_{90} could also be fractionated by n-butanol to yield S_{90}-Bu, a fraction enriched in antilipolytic activity.

The difference in slope between dose response curves of propranolol and S_{60} in inhibiting epinephrine-stimulated lipolysis, the inability of increasingly high doses of epinephrine to overcome the inhibition of S_{60}, and the ability of S_{60} to inhibit dbcAMP-induced lipolysis, all suggest that the saponin acts at a locus on the adipocytes other than the epinephrine receptor and beyond the generation of cAMP. The ineffectiveness of a high dose of epinephrine or corticotropin to reverse to a great extent the inhibition of S_{60} after a 2-hr incubation with adipocytes further supports the above conclusion.

The M. charantia seed saponin is also a noncompetitive inhibitor of corticotropin and glucagon in their lipolytic actions. The validity of these conclusions is based on the result that increasing the dose of corticotropin was ineffective in overcoming the inhibitory effect of propranolol. Propranolol is a β-adrenergic antagonist and competes with epinephrine for binding to the same membrane receptor, and it has been shown that epinephrine and corticotropin bind to different receptors on adipocyte membrane (Lang et al., 1976). Hence propranolol is a

noncompetitive inhibitor of corticotropin in its action on the adipocyte. This fact is well justified by the above experiment.

The antilipogenic activity of S^{60} was probably due to an inhibition of glucose transport into the adipocytes. These antilipolytic and antilipogenic property of the M. charantia seed saponin remind us of similar activities observed in ginsenosides (Ohminami et al., 1981) although they did not investigate into the mechanism of these inhibitory efects on lipolysis and lipogenesis. The viability of adipocytes after incubation with S^{60} under the assay conditions used was shown by its intrinsic, although slight, lipolytic activity at doses which inhibit hormone-induced lipolysis (Table 2-7), and also the normal appearance of the cells under the light microscope. This rules out the possibility that the antilipolytic and antilipogenic activities of the M. charantia seed saponin was in fact due to an adverse effect on cell viability.

2.4.4 Abortifacient protein and its chromatographically related fractions

Lipolytic activity was not detectable in α- and β-momorcharin (C_{5}-G_{1} and C_{6}-G_{1}), C_{5}-G_{2}, C_{6}-G_{2}, C_{5} and C_{6} at the dose tested (Table 2-13). In an assay to which a large number of samples together with C_{5} and C_{6} were assayed, C_{5} and C_{6} were incidentally left to incubate with corticotropin for 2-3 hours prior to addition of fat cells and the start of the assay. The apparent antilipolytic activity of C_{5} and C_{6} in this assay (Table 2-14) was not reproducible when the time that C_{5} and C_{6} had to react with corticotropin was minimized (Table 2-15). Alpha- and

Table 2-13

Assay of C_5, C_6 and their chromatographically fractions for lipolytic activity

Expt. No.	Fraction assayed	Dose (µg/tube)	Glycerol production (µmol/hr/g fat cell dry wt)
1	None (control)	-	1.32 ± 0.05
	C_5	100	1.37 ± 0.08 (NS)
	C_6	100	1.44 ± 0.06 (NS)
2	None (control)	-	2.05 ± 0.07
	C_5-G_1	30	1.98 ± 0.07 (NS)
	C_6-G_1	30	2.06 ± 0.05 (NS)
	C_5-G_2	30	2.04 ± 0.09 (NS)
	C_6-G_2	30	2.01 ± 0.10 (NS)

NS = Statistically not significant compared with control.

Table 2-14

Effects of C_5 and C_6 in inhibiting corticotropin-induced lipolysis

Antagonizing Fraction	Dose (μg/tube)	% of 25 mU ACTH-induced lipolysis
None	-	100
Propranolol	100	32.0 ± 1.4
	33	59.0 ± 3.3
	11	86.4 ± 1.3
C_5	100	2.4 ± 0.3
	20	45.9 ± 4.4
C_6	100	21.6 ± 8.5
	20	94.3 ± 3.1

ACTH (25 mU)elicited an net increase in glycerol production over the control at a rate of 4.76 ± 0.12 μmol glycerol per hr per g fat cell dry weight.

Table 2-15

Effects of C_5, C_6 and their derived fractions in inhibiting corticotropin-induced lipolysis

Antagonizing Fraction	Dose (μg/tube)	% of 25 mU ACTH-induced lipolysis
None (control)	-	100
Propranolol	100	20.0 ± 2.3
	33	56.1 ± 4.1
	11	91.6 ± 2.6
C_5	30	99.6 ± 2.1 (NS)
C_5-G_1	30	97.6 ± 0.8 (NS)
C_5-G_2	30	100.0 ± 4.7 (NS)
C_6	30	99.6 ± 0.8 (NS)
C_6-G_1	30	106.2 ± 3.1 (NS)
C_6-G_2	30	97.7 ± 3.5 (NS)

ACTH (25 mU) elicited an net increase in glycerol production over the control at a rate of 3.53 ± 0.05 μmol glycerol per hr per g fat cell dry weight.

NS = Statistically not significant compared with control.

beta-momorcharins, as well as C_{5-G_2} and C_{6-G_2}, were inactive (Table 2-15). In a subsequent assay the antilipolytic activities of C_5, and C_6 and AP, the starting material for purificaton of the abortifacient proteins, were examined at 0, 45, and 405 min after incubation with corticotropin prior to the start of the lipolysis assay. It was found that the apparent antilipolytic activities of AP, C_5, and C_6 seen at 405 min could not be observed at 0 and 45 min. (Table 2-16), confirming that the discrepancy between the results of the previous two assays (Table 2-14 & 2-15) was due to an interaction of C_5 and C_6 with corticotropin following in an impairment of the lipolytic potency of the hormone. The finding that C_5, C_6 and the abortifacient proteins derived from them lacked intrinsic antilipolytic activity (Table 2-15 & 2-16) was corroborated when the catecholamine, epinephrine, was used as the lipolytic agent (Table 2-17). Furthermore, antilipolytic activity was absent from the fractions chromatographically related to C_5 and C_6 i.e., C_1 to C_4 and C_7 and C_8 (Table 2-17). C_5 and C_6 had little inhibitory effect on glucagon-induced lipolysis although the effect of C_5 was statistically significant (Table 2-18). Both C_5 and C_6 did not exert any effect on the incorporation of tritiated glucose into lipid (Table 2-19).

The apparent ability of α- and β-momorcharin to inhibit corticotropin-stimulated lipolysis can be considered to be the result of an interaction with the hormone rather than to an interaction with receptors on the adipocytes since the phenomenon could be observed only when a certain lenght of time e.g. 2-3 hr

Table 2-16

Antilipolytic activities of AP, C_5 and C_6 after incubation with corticotropin for various time lengths at room temperature prior to incubation with adipocytes

ACTH dose (mU)	Antagonizing Fraction	Dose (μg/tube)	% of ACTH-induced lipolysis		
			t = 0 min	t = 45 min	t = 6 hr 45 min
25	None	-	100	100	100
25	Propranolol	100		-	23.0 ± 1.0
		33	-	70.5 ± 2.8	65.5 ± 2.1 (NS)
		11		91.6 ± 1.4	92.0 ± 2.7 (NS)
25	AP	500		85.3 ± 4.1	4.4 ± 1.5*
		100	-		63.1 ± 2.5
25	C_5	100	91.4 ± 1.8	108.9 ± 2.9	1.5 ± 0.8*
		30	98.3 ± 0.9	-	37.2 ± 1.0*
25	C_6	100	105.6 ± 2.2	109.5 ± 0.9	20.0 ± 2.0*
		30	100.7 ± 0.5	-	82.6 ± 1.0*
6.25	None	-	100	100	100
6.25	Propranolol	33		48.9 ± 3.5	
		11	-	77.1 ± 2.7	-
6.25	AP	500	64.1 ± 2.0		5.8 ± 0.8*
		100	-	-	29.3 ± 0.8
6.25	C_5	100	90.4 ± 2.9		
		30	106.3 ± 0.8	-	-
6.25	C_6	100	113.8 ± 0.8		
		30	115.5 ± 2.5	-	-

NS = no statistically significant difference, and * = $p < 0.001$, compared with the corresponding data at t = 0 min and t = 45 min.

ACTH elicited a net increase in glycerol production over the control at a rate of 2.78 ± 0.05 and 1.79 ± 0.01 μmol glycerol per hr per g fat cell dry weight at the doses 25 mU and 6.25 mU respectively.

Table 2-17

Effects of C_5, C_6 and chromatographically related fractions in inhibiting corticotropin- and epinephrine- induced lipolysis

Expt No.	Antagonizing Fraction	Dose (μg/tube)	% of 25 mU ACTH-induced lipolysis	% of 0.33 μg epinephrine bitartrate-induced lipolysis
1	Propranolol	1		7.3 ± 0.6
		0.3	-	16.7 ± 0
		0.1		27.9 ± 0.5
	C_5	20	-	120.3 ± 7.7
	C_6	20	-	125.9 ± 7.8
2	C_5	20	-	95.3 ± 1.5
		10	-	99.7 ± 0.9
	C_6	20	-	100.7 ± 0.3
		10	-	101.3 ± 1.5
3	C_5-G_1	200	-	110.3 ± 2.8
		100	-	107.3 ± 1.9
		50	-	111.3 ± 3.4
4	Propranolol	100	24.2 ± 1.0	
		33	67.5 ± 0.8	
		11	91.7 ± 3.4	
	C_1	250	104.8 ± 0.2	105.1 ± 1.9
	C_2	250	119.4 ± 2.8	111.2 ± 1.1
	C_3	250	125.2 ± 2.4	120.0 ± 2.3
	C_4	250	113.2 ± 1.7	107.8 ± 1.0
	C_5	250	116.9 ± 2.0	109.4 ± 0.8
	C_6	250	119.0 ± 0.4	100.9 ± 2.3
	C_7	250	110.4 ± 1.0	99.1 ± 0.4
	C_8	250	107.0 ± 1.3	102.7 ± 2.2

ACTH (25 mU) elicited an increase of glycerol production over the control at a rate of 4.29 ± 0.08 μmol glycerol per hr per g fat cells dry weight. The corresponding value for epinephrine-induced lipolysis ranged from 2.02 ± 0.03 to 2.86 ± 0.04 in experiments 1, 2, 3 and 4.

Table 2-18

Effects of C_5 and C_6 in inhibiting glucagon-induced lipolysis.

Antagonizing Fraction	Dose (μg/tube)	% of 0.2 μg glucagon-induced lipolysis
None	-	100
Propranolol	100	6.8 ± 1.0
	33	47.6 ± 1.8
	11	78.7 ± 1.8
C_5	500	90.9 ± 1.0 ($p < 0.025$)
	250	108.8 ± 0.3
C_6	500	111.9 ± 3.2
	250	124.7 ± 5.9

Glucagon (0.2 μg) elicited net increase in glycerol production over the control at a rate of 1.73 ± 0.04 μmol glycerol per hr per g fat cell dry weight.

Table 2-19

Lipogenic activities of C_5 and C_6.

Fraction	Dose	D-[3-3H]-glucose incorporated into lipid (cpm)
Control	-	2066 ± 163
Insulin	1000 μU	6253 ± 84
	10 μU	3532 ± 199
C_5	100 μg	1868 ± 45 (NS)
	20 μg	1800 ± 24 (NS)
C_6	100 μg	1886 ± 88 (NS)
	20 μg	1900 ± 21 (NS)

D-[3-3H]-glucose (84130 ± 842 cpm), and around 2 x 10^4 fat cells (3.56 mg total lipid) were added to each assay vial.

NS = Statistically not significant compared with control.

or 7 hr was allowed for interacton of the abortifacient proteins with corticotropin to occur *in vitro*, prior to the additon of adipocytes and commencement of the assay. The observation was not reproducible when the proteins and the peptide hormone were introduced into the fat cells immediately or shortly before the beginning of the incubation, or when the lipolytic stimulus was provided by the catecholamine, epinephrine, in lieu of corticotropin. The nature of the interaction of α- and β-momorcharins with corticotropin which follows in a drastic reduction in the lipolytic potency of the hormone remains to be elucidated. One possibility is that the active core of the hormone, comprising the amino acids sequence Met-Glu-His-Phe-Arg-Trp-Gly (Li, 1972) deformed by such interaction, and consequently prevented from expressing its lipolytic activity which is dependent on the integrity of this core sequence. Further speculations are inappropriate at this stage.

Alpha- and beta-momorcharins are devoid of lipolytic, antilipolytic and lipogenic activities. Since insulin exerts its effects on lipolysis and lipogenesis in adipose tissue by affecting glucose uptake and activities of enzymes (Tepperman, 1975), it may be implied that the momorcharins do not influence glucose uptake by the adipocytes and the activities of adipocyte enzymes regulating lipolysis and lipogenesis.

2.4.5 Fruit and seed "p-peptides"

The fruit "p-peptide" gave a negative reaction in the Liebermann test and hemolysis test. Protein bands were stainable after sodium dodecyl sulfate polyacrylamide gel electrophoresis

and agarose electrophoresis. Its ultraviolet absorption spectrum revealed a maximum at 262 nm (data from our laboratory).

The fruit "p-peptide" lacked any significant effect in inhibiting lipolysis induced by epinephrine, corticotropin, glucagon and dibutyryl cyclic AMP in rat adipocytes (Table 2-20). However, it exhibited antilipolytic activity in hamster adipocytes (Table 2-20) and lipogenic activity in rat adipocytes (Table 2-21).

The seed "p-peptide" gave a negative Liebermann test, an ultraviolet absorption spectrum similar to that of insulin and two to three diffuse protein bands with a molecular weight less than 20,000 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, the presence of saponin(s) in the preparation was indicated by a positive reaction in the hemolysis assay and a saponin spot in its thin layer chromatogram (data from our laboratory).

The seed "p-peptide" had potent antilipolytic activity in rat adipocytes (Table 2-22). The slope of its inhibiton curve in an epinephrine-stimulated lipolysis was much steeper than those of propranolol and insulin (Figure 2-30). Increasing the doses of epinephrine, glucagon or corticotropin was inefficacious in overcoming the inhibitory effect of 250 µg seed "p-peptide" on lipolysis stimulated by these hormones respectively (Figure 2-31, 32 & 33). Treatment of seed "p-peptide" with trypsin, chymotrypsin, pronase, emulsin and glutathione did not affect its antilipolytic potency. The antilipolytic activity of 200 µg seed "p-peptide" was abolished by heating while that of a bigger dose

Table 2-20

Effects of fruit "p-peptide" in inhibiting epinephrine, corticotropin, glucagon-, and dibutyrl cAMP-induced lipolysis

Expt. No.	Assay Animal	Antagonizing fraction	Dose (μg/tube)	% lipolysis induced by			
				Epinephrine (0.33 μg)	ACTH (25 mU)	Glucagon (0.2 μg)	Dbc-AMP (2 mM)
1	Rat	None	—	100	100	—	—
		Insulin	100 μU	20.7 ± 1.0	—		
			10 μU	40.0 ± 0.3	55.2 ± 1.3		
			1 μU	86.9 ± 1.5	75.0 ± 2.5		
			0.1 μU	99.4 ± 1.3	85.0 ± 1.5		
		Fruit "p-peptide"	500 μg	110.8 ± 3.4	101.1 ± 6.2		
			250 μg	130.0 ± 2.1	88.1 ± 2.4		
			100 μg	132.5 ± 2.8	91.6 ± 1.5		
			50 μg	125.1 ± 1.6	67.1 ± 4.6		
			25 μg	122.2 ± 2.6	89.6 ± 4.9		
2		None	—	100	100	100	100
		Fruit "p-peptide"	500 μg	89.7 ± 3.1	101.7 ± 0.6	104.5 ± 2.3	101.23 ± 0.37
			250 μg	91.5 ± 3.9	106.8 ± 0.3	100.4 ± 4.4	98.37 ± 2.14
			100 μg	93.0 ± 6.6	114.5 ± 2.0	97.2 ± 1.3	—
3	Hamster	None	—	—	100[a]	—	—
		Fruit "p-peptide"	500 μg		0		
			250 μg		83.71 ± 2.28		

a: 0.5 mU ACTH was used instead of 25 mU in the case of hamster adipocytes

Table 2-21

Lipogenic activity of fruit "p-peptide"

Expt. No.	Fraction	Dose	% of added counts per 10 mg lipid
1	None	—	0
	Insulin	1000 μU	11.20 ± 0.33
	Fruit	500 μg	16.14 ± 6.4
	"p-peptide"	100 μg	10.04 ± 0.0
		20 μg	9.04 ± 5.58
2	None	—	0
	Insulin	1000 μU	7.42 ± 0.15
	Fruit	500 μg	2.16 ± 0.79
	"p-peptide"	100 μg	0.14 ± 0.02

In experiment 1 83837 ± 2409 cpm D-[3-3H]glucose and about 1.1×10^5 adipocytes were added to each assay tube. In experiment 2 37743 ± 292 cpm D-[3-3H]glucose and about 1.1×10^5 adipocytes were added per assay tube.

Table 2-22

Effects of seed "p-peptide" in inhibiting epinephrine-, corticotropin-, glucagon, and dibutyryl cAMP-induced lipolysis

Expt. No.	Antagonizing fraction	Dose (μg/tube)	% lipolysis induced by			
			Epinephrine (0.33 μg)	ACTH (25 mU)	Glucagon (0.3 μg)	Dbc AMP (2 mM)
1	None	—	100	100	100	100
	Seed "p-peptide"	500	1.6 ± 0.1	1.7 ± 0.2	1.04 ± 0.16	5.71 ± 0.97
		250	4.7 ± 0.2	4.0 ± 0.1	13.28 ± 0.88	24.02 ± 1.38
		100	71.0 ± 2.8	93.4 ± 5.5	—	93.78 ± 4.32
2	None	—	100	100	—	—
	Seed "p-peptide"	500	5.6 ± 0.1	3.1 ± 0.6		
		250	12.2 ± 0.4	6.0 ± 1.0		
		100	74.6 ± 2.1	87.2 ± 2.7		
		50	109.0 ± 0.7	108.4 ± 1.8		

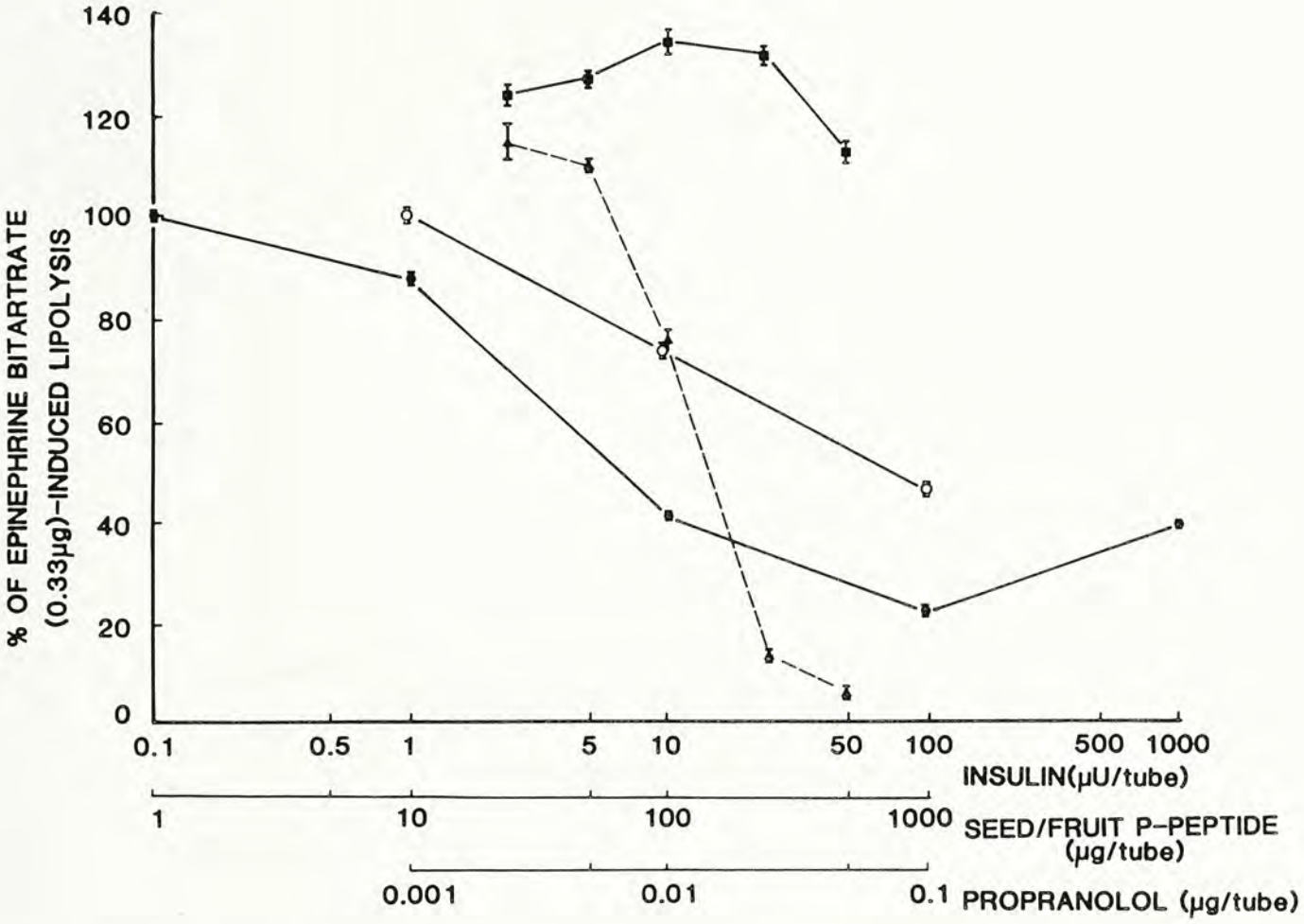


Figure 2-30. Effects of fruit and seed "p-peptides" on epinephrine bitartrate (0.33μg)-induced glycerol production. The fruit "p-peptide" (■) did not inhibit lipolysis. The seed "p-peptide" (▲---▲) had strong antilipolytic activity. The slope of its inhibition curve was much steeper than those of propranolol (○) and insulin (●).

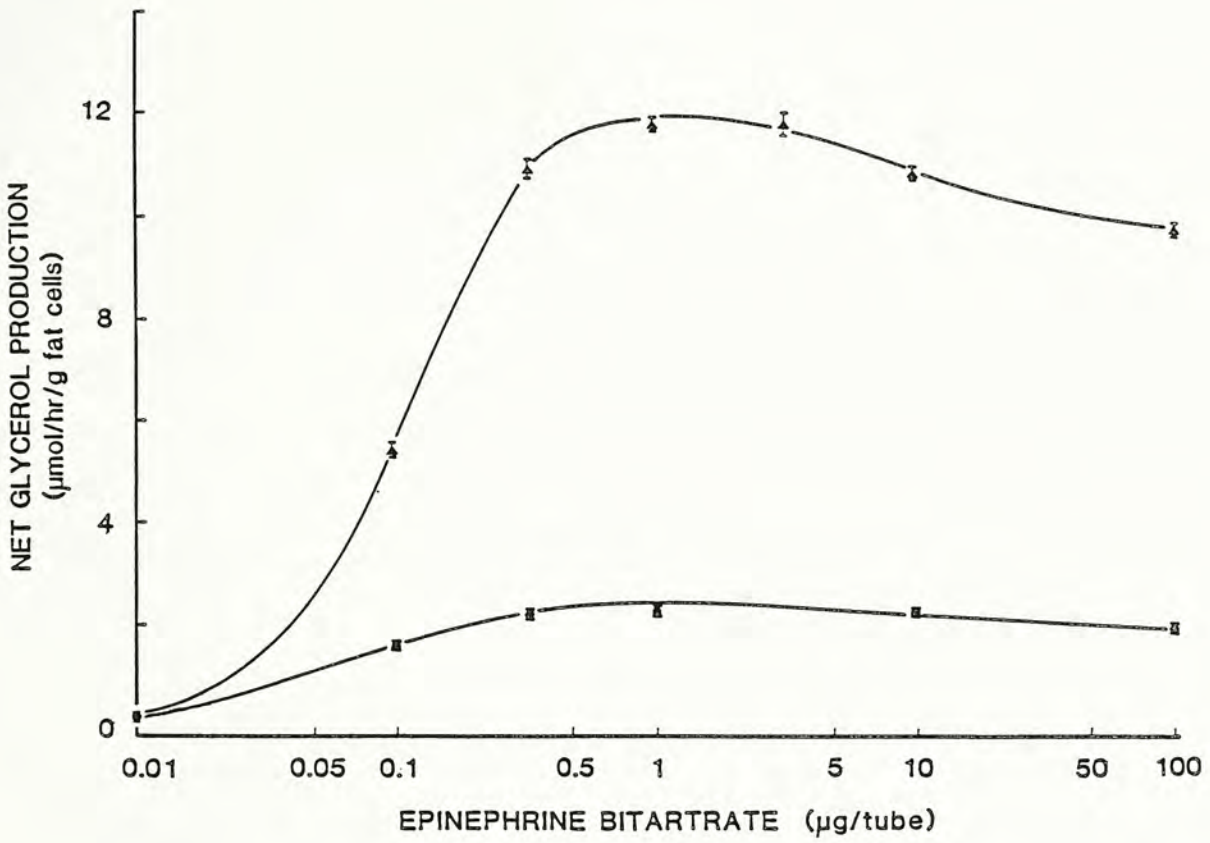


Figure 2-31. Effect of increasing doses of epinephrine (▲) on the antilipolytic activity of seed "p-peptide" (●). The inhibitory effect of seed "p-peptide" could not be overcome by increasing the dose of the hormone. Seed "p-peptide" was added at a dose of 250 µg/tube.

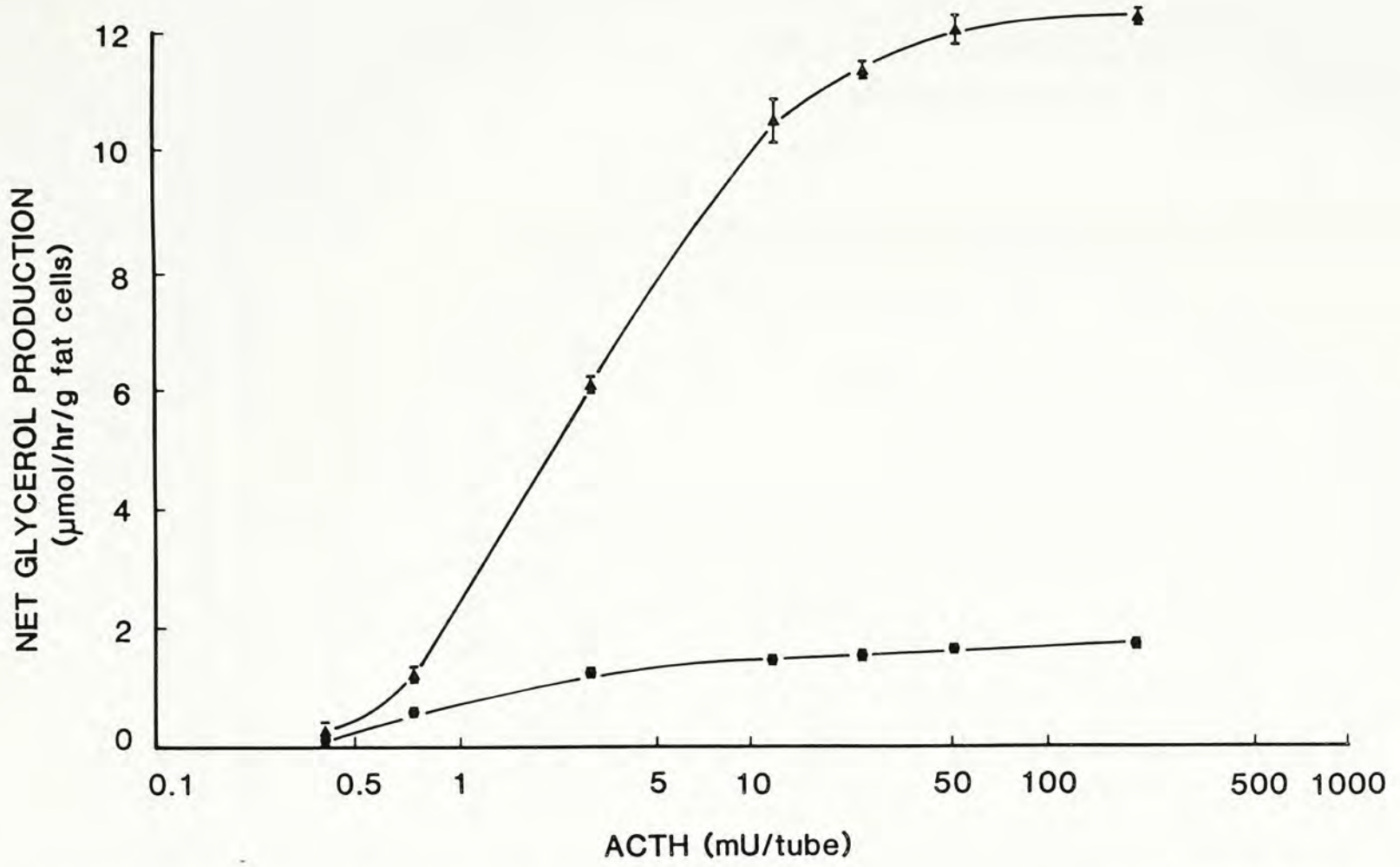


Figure 2-32. Effect of increasing dose of ACTH (▲) on the antilipolytic activity of seed "p-peptide" (●). The inhibitory effect of seed "p-peptide" could not be overcome by increasing the dose of the hormone. Seed "p-peptide" was added at a dose of 250 µg/tube.

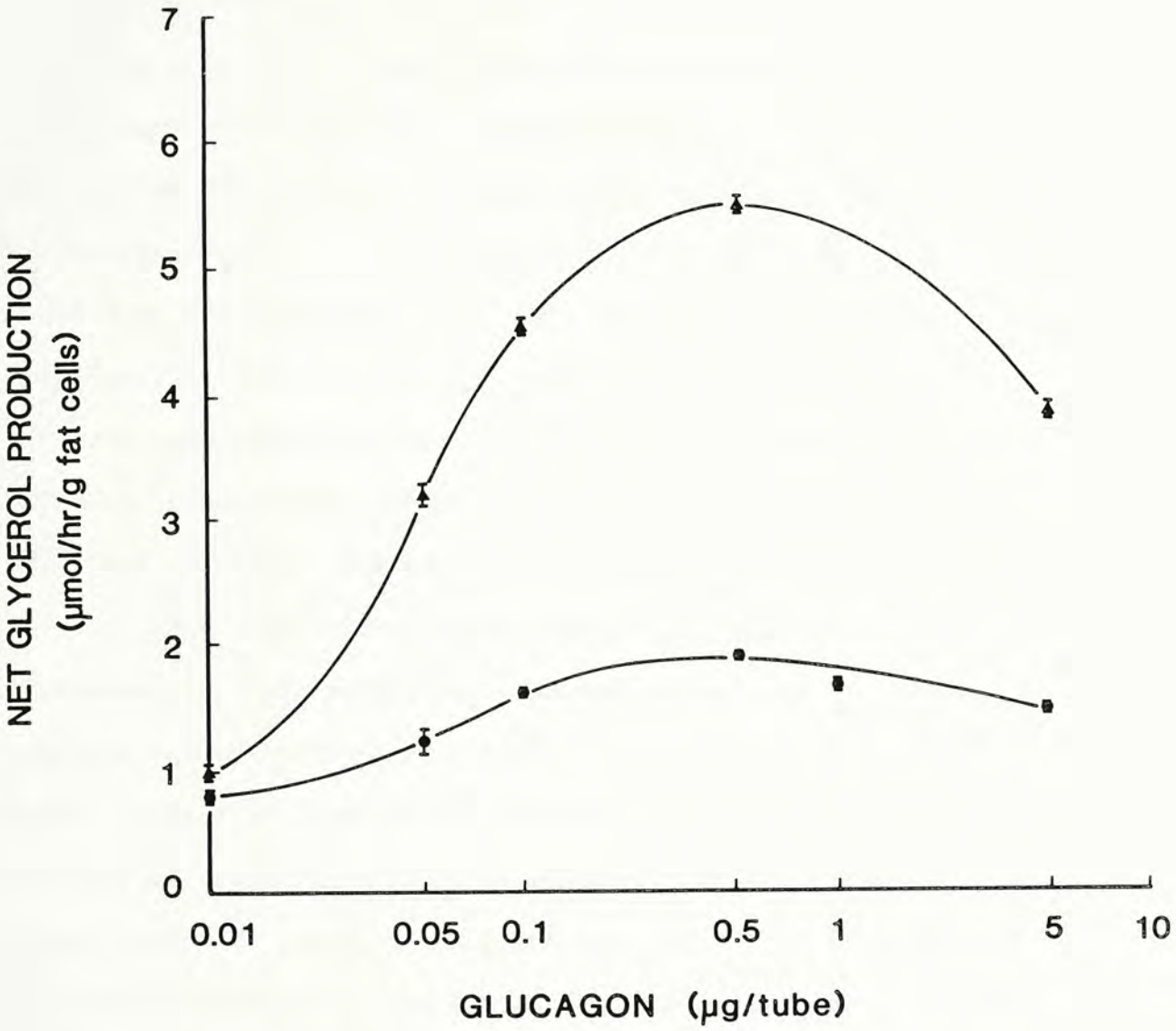


Figure 2-33. Effect of increasing dose of glucagon (▲) on the antilipolytic activity of seed "p-peptide" (●). The inhibitory effect of seed "p-peptide" could not be overcome by increasing the dose of the hormone. Seed "p-peptide" was added at a dose of 250 µg/tube.

(500 µg) was only partially destroyed (Table 2-23). Seed "p-peptide" also exhibited antilipogenic activity which was not destroyed by heating (Table 2-24).

Affinity chromatography of 30 mg seed "p-peptide" on fetuin agarose followed by gel filtration of the unadsorbed fraction on Sephadex G-10 yielded a large peak in the void volume (p-peptide-F_1, 24.5 mg) and two small retarded peaks (p-peptide-F_2, 1.5 mg and p-peptide-F_3, 1.5 mg). P-peptide-F_1 possessed both antilipolytic and antilipogenic activities and the antilipolytic activity was heat resistant. P-peptide-F_2 possessed lipogenic activity (Table 2-25). There was not enough p-peptide-F_2 and p-peptide-F_3 for heat treatment and antilipolysis assay.

The fruit "p-peptide" exerted a stimulatory effect on lipogenesis but had no effect on hormone-stimulated lipolysis in isolated rat adipocytes. It inhibited corticotropin-induced lipolysis in hamster adipocytes, however. This discrepancy in the activity of fruit "p-peptide" in adipocytes from the two different rodent species tends to suggest that the fruit "p-peptide" inhibited lipolysis in hamster adipocytes by interacting either with the corticotropin receptor or with the alpha-adrenergic receptor which is absent from rat adipocytes (Carpere et al., 1983).

The seed "p-peptide" possessed antilipolytic and antilipogenic activity. The inhibition curve of seed "p-peptide" had a slope different from that of the propranolol inhibition curve in the epinephrine-stimulated lipolysis assay. Furthermore, the seed "p-peptide" was capable of inhibiting dibutyryl cyclic AMP-stimulated lipolysis. These findings suggest that the site of

Table 2-23

Effect of heat and enzymes on antilipolytic activity of seed "p-peptide"

	Treatment of seed "p-peptide"	Glycerol production (μmol per hr per g fat cell dry weight)
Buffer	N.A.	0
Epinephrine (control)	N.A.	15.17 ± 0.25
Epinephrine + p-peptide (250 μg)	None	3.28 ± 0.03[a]
Epinephrine + p-peptide (500 μg)	None	2.85 ± 0.01[a]
Epinephrine + p-peptide (250 μg)	Heat	16.82 ± 0.61[b]
Epinephrine + p-peptide (500 μg)	Heat	5.70 ± 0.07[a]
Epinephrine + p-peptide (500 μg)	Trypsin	2.85 ± 0.01[a]
Epinephrine + p-peptide (500 μg)	Chymotrypsin	2.92 ± 0.04[a]
Epinephrine + p-peptide (500 μg)	Pronase	3.06 ± 0.03[a]
Epinephrine + p-peptide (500 μg)	Emulsin	2.88 ± 0.03[a]
Epinephrine + p-peptide (500 μg)	Glutathione	2.94 ± 0.03

N.A. = not applicable

Dose of epinephrine was 0.33 μg throughout.

a = $p < 0.001$ compared with control, b = statistically not significant compared with control.

Table 2-24

Antilipogenic activity of seed "p-peptide"

Fraction	Dose (µg/tube)	D-[3-3H]-glucose incorported into lipid (cpm)
None	—	1949 ± 146
Seed "p-peptide"	500	188 ± 1 ($p < 0.001$)

D-[3-3H]-glucose (83837 ± 2409 cpm) and 1.1×10^5 cells were added to each assay tube.

Table 2-25

Effect of fractions derived from seed "p-peptide" after affinity chromatography on fetuin agarose on lipolysis and lipogenesis.

Fraction	Dose	% of 0.33ug epinephrine bitartrate-induced lipolysis[a]	D-[3-3H]-glucose incorporated into lipid[b] (cpm)
None (control)	-	100	8533 ± 314
Insulin	1 mU	-	25637 ± 780[c]
p-peptide	500 µg	1.79 ± 0.07	168 ± 1 [c]
	250 µg	15.08 ± 1.04	-
p-peptide+heat	500 µg	2.87 ± 0.07	175 ± 4 [c]
	250 µg	12.59 ± 0.25	-
p-peptide-F_1	500 µg	2.12 ± 0	157 ± 1 [c]
	250 µg	15.16 ± 1.42	-
p-peptide-F_1+heat	500 µg	2.26 ± 0.27	162 ± 2 [c]
	250 µg	14.83 ± 1.17	-
p-peptide-F_2	500 µg	-	12237 ± 152[c]
p-peptide-F_3	500 µg	-	4032 ± 46 [c]

a : Epinephrine bitartrate (0.33 µg) elicited a net increase in glycerol production over the control at a rate of 11.82 ± 0.33 µmole glucerol per hr per g fat cell dry weight.

b : D- [3-3H]-glucose (98256 ± 660 cpm) and 1.3×10^5 cells were added to each assay vial.

c : $p < 0.001$ when compared with control

action of the seed "p-peptide" is beyond the generation of cyclic AMP, the intracellular messenger for lipolytic hormones. Increasing the dose of epinephrine was ineffective in reversing the antilipolytic effect of the "p-peptide". Seed "p-peptide" acts as a noncompetitive inhibitor of epinephrine. A similar phenomenon was observed when glucagon or corticotropin was used as the lipolytic agent. Based on these evidence,it can be concluded that the seed "p-peptide" did not exert its antilipolytic activity by competing with the lipolytic hormones for binding to their respective receptors on the adipocyte plasma membrane. Its antilipogenic activity was probably due to a suppressive effect on glucose uptake. The resistance of its antilipolytic activity to proteases such as trypsin and chymotrypsin and only partial destruction by heat infer the existence of a heat-stable non-protein component. Such component may be a saponin(s) as shown by positive reaction in the hemolysis assay and the Liebermann's test. The percentage of heat labile antilipolytic activity may be due to a protein component which can be revealed by subjecting the seed "p-peptide" to SDS-polyacrylamide gel electrophoresis and afterwards staining the electrophoretic bands with Coomassie-blue. Due to the heterogeneity of the seed "p-peptide" preparation we attempted to separate the saponin component from the protein component by affinity chromatography on fetuin agarose. The saponin component(s) were unadsorbed on the immobilized lectin and were eluted as the first peak after gel filtration on Sephadex G-10 (p-peptide-F_1). The dose response and heat lability of the antilipolytic activity of this peak was

similar to that of the parent seed "p-peptide". In fact, it was the major component of seed "p-peptide" and possessed essentially all of the antilipolytic and antilipogenic activities observed in seed "p-peptide". The hemolytic activity of seed "p-peptide" was also concentrated in this peak. This peak may contain protein in addition to saponin(s) on account of its high absorbance at 280 nm (Data from our laboratory). The second peak derived by chromatographing the unadsorbed peak from the fetuin agarose column on Sephadex G-10 (p-peptide-F_2) was obtained in a much lower yield than the first peak. Lipogenic activity was present in this peak but unfortunately the amount of material remaining did not permit studies of its antilipolytic activity and chemical purity. It is likely that the heat labile antilipolytic activity was attributed to p-peptide-F_2.

The observation of antilipolytic (in hamster system) and lipogenic activities in fruit "p-peptide" and lipogenic activity in a fraction derived from seed "p-peptide" indicated the presence in M. charantia fruits and seeds, compounds that could be extracted by acid-ethanol and exhibited insulinomimetic activities in vitro. This finding showed some correlation with the demonstration of hypoglycemic activity of similarly prepared fractions by Khanna et al (1981).

2.4.6 Insulin-like peptide(s) isolated by the insect insulin purification scheme

After its pH had been adjusted to neutrality, the supernatant obtained from acid ethanol extraction of the seeds was separated into a precipitate called P_1 and a yellowish

supernatant (Chart 4). The yellowish supernatant was further fractionated by gel filtration on Sephadex G50 into four fractions, S_1, S_2, S_3 and S_4. P was antilipogenic (Table 2-27). S_1, S_2 and S_3 inhibited both lipogenesis and lipolysis with a dose response curve similar to that of saponin illustrated previously (Table 2-26, 27). S_4 resembled insulin in that it had both lipogenic and antilipolytic activities. After CM Sepharose chromatography, seven fractions, IC-1 to IC-7 were obtained from S_4. IC-1 was both antilipolytic and antilipogenic. IC-2 possessed only antilipolytic activities (Table 2-28 and 2-29). Both fractions had saponin activity as revealed by their hemolytic activity (data from our laboratory). IC-3 to IC-7 were devoid of any antilipolytic effect in the rat adipocyte system. However IC-3 exhibited a suppressive effect on corticotropin-induced lipolysis in hamster adipocytes (Table 2-30). Thus, the apparent antilipolytic activity of S_4 may be due to the antilipolytic activities present in IC-1 and IC-2 whose antilipogenic activities were masked by the lipogenic effect of IC-3, IC-4, IC-5 and IC-7. The disparity in the finding on adipocytes from the two rodent species suggests that IC-3 to IC-7 do not act as β-adrenergic antagonists but may inhibit lipolysis in hamster adipocytes by interacting with the corticotropin receptor or the α-adrenergic receptors. None of the fractions was pure and some of them contained many nonproteinaceous compounds as checked by agarose electrophoresis. So in view of the shortage of time, further purification of the fractions was not attempted. However, it can still be concluded that IC-3, IC-4 and IC-5 contained some

Table 2-26

Antilipolytic effect of fractions isolated by insect insulin purification scheme.

Antagonizing fraction	Dose (µg/tube)	% of 0.33 µg epinephrine bitartrate-induced lipolysis
None	-	100
S_1	500	5.2 ± 1.5
	250	3.1 ± 2.6
	100	17.8 ± 1.4
S_2	500	8.1 ± 0.7
	250	13.2 ± 2.6
	100	90.1 ± 0.5
S_3	500	53.9 ± 1.7
	250	82.2 ± 1.4
	100	107.1 ± 2.4
S_4	500	78.9 ± 1.2
	250	88.6 ± 1.1
	100	110.8 ± 4.0

Epinephrine bitartrate (0.33 µg) elicited an increase of glycerol production over the control at a rate of 5.51 ± 0.07 µmole glycerol per hr. per g fat cell dry weight.

Table 2-27

Lipogenic activities of fractions isolated by insect insulin purification scheme.

Fraction	Dose	D-[3-3H]-glucose incorporated into lipid (cpm)
Control	-	2059 ± 46
Insulin	1000 μU	2984 ± 39 ($p < 0.005$)[a]
	500 μU	2598 ± 34 ($p < 0.01$)
P_1	500 μg	476 ± 20 ($p < 0.001$)
S_1	500 μg	169 ± 3 ($p < 0.001$)
S_2	500 μg	172 ± 4 ($p < 0.001$)
S_3	500 μg	585 ± 27 ($p < 0.001$)
S_4	500 μg	3990 ± 110 ($p < 0.001$)

D-[3-3H]-glucose (91,401 ± 408 cpm), and 9.1×10^4 fat cells were added to each assay vial.

a : Compared with control.

Table 2-28

Lipogenic activities of the fractions isolated by insect insulin purification scheme

Fraction	Dose	D-[3-3H]-glucose incorporated into lipid (cpm)	
Control	-	3076 ± 57	
Insulin	10 mU	5140 ± 152	
IC-1	250 µg	183 ± 5	
IC-2	50 µg	3137 ± 47	(NS)
IC-3	500 µg	3816 ± 130	($p < 0.005$)[a]
	250 µg	3618 ± 34	($p < 0.05$)
IC-4	500 µg	4333 ± 540	($p < 0.0025$)
	250 µg	4050 ± 15	($p < 0.005$)
IC-5	500 µg	3687 ± 122	($p < 0.05$)
	250 µg	4101 ± 159	($p < 0.005$)
IC-6	500 µg	2672 ± 250	(NS)
	250 µg	2814 ± 31	(NS)
IC-7	500 µg	4710 ± 207	($p < 0.001$)
	250 µg	4824 ± 83	($p < 0.001$)

D-[3-3H]- glucose (8,0116 ± 1048 cpm)&about 9 x 10^4 fat cells were added to each assay vial.

a : Compared with control.

NS = Statistically not significant compared with control.

Table 2-29

Effects of fractions isolated by the insect insulin purification scheme in inhibiting epinephrine-induced lipolysis in rat adipocytes

Antagonizing fraction	Dose (μg/tube)	% of 0.33 μg epinephrine bitartrate-induced lipolysis
None	-	100
IC-1	250	3.20 ± 0.20
IC-2	200	61.12 ± 3.19
IC-3	500	105.37 ± 2.21
	250	102.00 ± 4.09
IC-4	500	108.77 ± 2.33
	250	111.03 ± 3.94
IC-5	500	108.37 ± 6.53
	250	114.39 ± 1.42
IC-6	500	100.49 ± 4.66
	250	107.31 ± 5.17
IC-7	500	128.12 ± 0.97
	250	123.52 ± 2.92

Epinephrine bitartrate (0.33 μg) elicited a net increase of glycerol production over the control at a rate of 13.41 ± 0.44 μmole per hr per g fat cell dry weight.

Table 2-30

Effect of fractions isolated by insect insulin purification scheme in inhibiting ACTH-induced lipolysis in hamster adipocytes

Antagonizing fraction	Dose (μg/tube)	% of 0.5 mU ACTH-induced lipolysis in hamster adipocytes
None	-	100
IC-3	300	24.84 ± 3.20
IC-4	300	74.91 ± 3.32
IC-5	300	63.96 ± 2.35
IC-6	300	0
IC-7	300	133.08 ± 5.38

There was no enough IC-1 and IC-2 for test.

insulin-like components because these fractions exhibited both antilipolytic and lipogenic activities. Further experiments are needed to elucidate the relationship among these components.

2.4.7 Insulin-like peptide(s) isolated by the mammalian insulin purification scheme

It can be seen from Tables 2-31 and 2-32 that fraction P_2 had both antilipolytic and antilipogenic activities similar to *M. charantia* saponin. P_1 was lipogenic though it lacked antilipolytic activity. So P_1 instead of P_2 was used for further purification of insulin-like peptide(s). The antilipolytic and lipogenic activities of the fractions derived from P_1 after CM-Sepharose CL-6B chromatography were presented in Tables 2-33 and 2-34 respectively. All fractions except C_{1-1}, C_{2-2} and C_{2-3} possessed antilipolytic and lipogenic activities. C_{1-1} and C_{2-1} had hemolytic activity. C_{2-3} contained nonproteinaceous components. All fractions except C_{1-3} and C_{6-1} were not pure as they could be separated into more than one spot in agarose electrophoresis. As judged from mobility in agarose electrophoresis, C_{1-3} behaved similarly to insulin i.e. it was an acidic protein, whereas C_{6-1} was a basic protein (data from our laboratory). Both C_{1-3} and C_{6-1} were heat labile (Table 2-35). The purified insulin like fractions C_{1-3} and C_{6-1} were then tested for their effects on plasma glucose and total lipid level in fasting aged rats.

In anaesthesized state,the mean plasma glucose and total lipid levels of aged rats were 100.10±1.84 mg/dl (26 rats)

Table 2-31

Effect of P_1 and P_2 isolated by mammalian insulin purification scheme on epinephrine-induced lipolysis

Antagonizing fraction	Dose (µg/tube)	% of 0.33 µg epinephrine bitartrate- induced lipolysis
None	-	100
P_1	500	113.1 ± 1.2
	250	123.9 ± 11.1
	100	134.5 ± 5.4
P_2	500	88.7 ± 2.4
	250	184.0 ± 5.8
	100	173.6 ± 1.1

Epinephrine bitartrate (0.33 µg) elicited an increase of glycerol production over the control at the rate of 5.51 ± 0.07 µmole per hr per g fat cell dry weight.

Table 2-32

Lipogenic activities of P_1 and P_2 isolated by mammalian insulin purification scheme

Fraction	Dose	D-[3-3H]-glucose incorporated into lipid (cpm)	
None (control)	-	2059 ± 46	
Insulin	1000 µU	3022 ± 19	($p < 0.005$)[a]
P_1	500 µg	4074 ± 123	($p < 0.001$)
P_2	500 µg	272 ± 20	($p < 0.001$)

D-[3-3H]-Glucose (91,401 ± 408 cpm) and 9.1×10^4 fat cells were added to each assay vial.

a : Compared with control.

Table 2-33

Effectsof fractions isolated by mammalian insulin purification scheme on epinephrine-induced lipolysis in rat adipocytes.

Expt. No.	Antagonizing fraction	Dose (µg/tube)	% of epinephrine bitartrate (0.33 µg)-induced lipolysis
1	None	-	100
	C_{1-1}	500	12.44 ± 0.24
		250	16.98 ± 0.92
	C_{1-3}	500	78.93 ± 0.23
		250	86.53 ± 2.09
	C_{2-1}	500	15.9 ± 0.54
		250	29.69 ± 0.90
	C_{2-2}	500	90.21 ± 2.53
		250	85.94 ± 1.01
	C_{2-3}	500	85.58 ± 3.09
		250	96.36 ± 1.65
	C_{3-1}	500	14.73 ± 0.20
		250	29.34 ± 0.99
	C_{4-1}	500	25.70 ± 1.22
		250	52.97 ± 2.24
	C_{5-1}	500	23.09 ± 0.58
		250	46.0 ± 1.80
	C_{6-1}	500	29.20 ± 0.86
		250	55.75 ± 1.47
	C_{7-1}	500	97.49 ± 0.86
		250	101.89 ± 1.60
2	C_{1-3}	250	78.65 ± 1.84
	C_{6-1}	500	31.69 ± 0.09
		250	65.00 ± 1.01

Epinephrine bitartrate (0.33 µg) elicited an increase of glycerol production over the control at the rate of 13.54 ± 0.4 and 4.29 ± 0.11 µmole per hr per g fat cell dry weight in experiment 1 and 2 respectively.

Table 2-34

Lipogenic activities of fractions isolated by mammalian insulin purification scheme

Fraction	Dose	% of added counts/10 mg lipid	
		Expt. 1	Expt. 2
None	-	0	0
Insulin	1 mU	27.19 ± 0.57	21.09 ± 0.98
C_{1-1}	200 µg	27.95 ± 0.56	23.9 ± 0.24
	40 µg	13.30 ± 0.22	-
C_{1-3}	200 µg	27.01 ± 0.7	25.55 ± 1.07
	40 µg	8.33 ± 0	-
C_{2-1}	200 µg	27.70 ± 0.30	24.14 ± 1.13
	40 µg	9.31 ± 0.27	-
C_{2-2}	200 µg	1.10 ± 0.09	-
	40 µg	0.00029	-
C_{2-3}	200 µg	1.91 ± 0.25	0.0004
	40 µg	1.01 ± 0.10	-
C_{3-1}	200 µg	25.75 ± 0.01	21.78 ± 0.38
	40 µg	6.44 ± 0.09	-
C_{4-1}	200 µg	21.92 ± 0.44	21.23 ± 0.97
	40 µg	3.81 ± 0.13	-
C_{5-1}	200 µg	25.01 ± 0.40	23.78 ± 0.54
	40 µg	7.13 ± 0.63	-
C_{6-1}	200 µg	21.78 ± 0.1	20.60 ± 0.43
	40 µg	4.36 ± 0.19	-
C_{7-1}	200 µg	0.97 ± 0.44	0.64 ± 0.54
	40 µg	0.57 ± 0.44	-

D-[3-3H]-Glucose (97,159 ± 435 and 98256 ± 660 cpm) and fat cells containing 5.82 and 8.26 mg lipid were added to each vial in experiment 1 and 2 respectively.

Table 2-35

Effect of heat treatment on the lipogenic activities of fractions isolated by mammalian insulin purification scheme

Fraction	Dose	% of added counts/ 10 mg lipid
None	-	0
Insulin	1 mU	27.19 ± 0.57
C_{6-1}	200 µg	21.78 ± 0.1
C_{6-1}-H	200 µg	2.87 ± 0.25
C_{1-3}	200 µg	27.01 ± 0.7
C_{1-3}-H	200 µg	1.03 ± 0.35

D-[3-3H]-Glucose (97,159 ± 435 cpm) and fat cells containing 5.82 mg lipid were added to each assay vial.

C_{6-1}-H and C_{1-3}-H denote the heat treated C_{6-1} and C_{1-3} fractions respectively.

and 280.67±12.27 mg/dl (26 rats) respectively, while the corresponding values of fasting young rats were 69.55±0.63 mg/dl (10 rats) and 181.86±5.8 mg/dl (10 rats) respectively (both values have $p < 0.001$ when compared with aged rats). Both hyperglycemia and hyperlipidemia are metabolic alternations found in diabetes. Old rats were therefore used, as mentioned in general introduction, as diabetic model to test for the possible hypoglycemic effects of the insulin like proteins C_{1-3} and C_{6-1}.

Insulin injected subcutaneously at a dose of 1 U/Kg greatly decreased the plasma glucose level. The maximal fall was seen 2 hour postinjection and the blood glucose level started to rise back up slowly from 2 hour onwards (Figure 2-34). Subcutaneous administration of 2 mg/Kg dose of C_{1-3} and C_{6-1}, however, produced no significant hypoglycemic effect on fasting old rats. C_{6-1} still could not appreciably lower the blood glucose level even when tested to a dose of 5 mg/Kg. There was not enough material to test either sample at a higher dose.

Owing to the fact that the difference in plasma total lipid level between individual aged rats was fairly great (ranging from 200 to 400 mg/dl), it is necessary to compare the postinjection values with that at preinjection value (0 hour) within each group of rat. Figure 2-35 shows that the mean plasma total lipid level of control, C_{6-1} treated and C_{1-3} treated groups at 1 to 6 hour postinjection was not significantly lower ($p > 0.005$) than that at 0 hour while insulin caused a significant ($p < 0.05$) decrease at 3 and 4 hour intervals. Although there was a significant fall in plasma total lipid in both C_{6-1} and C_{1-3} treated groups at 7 and 8 hour intervals, it

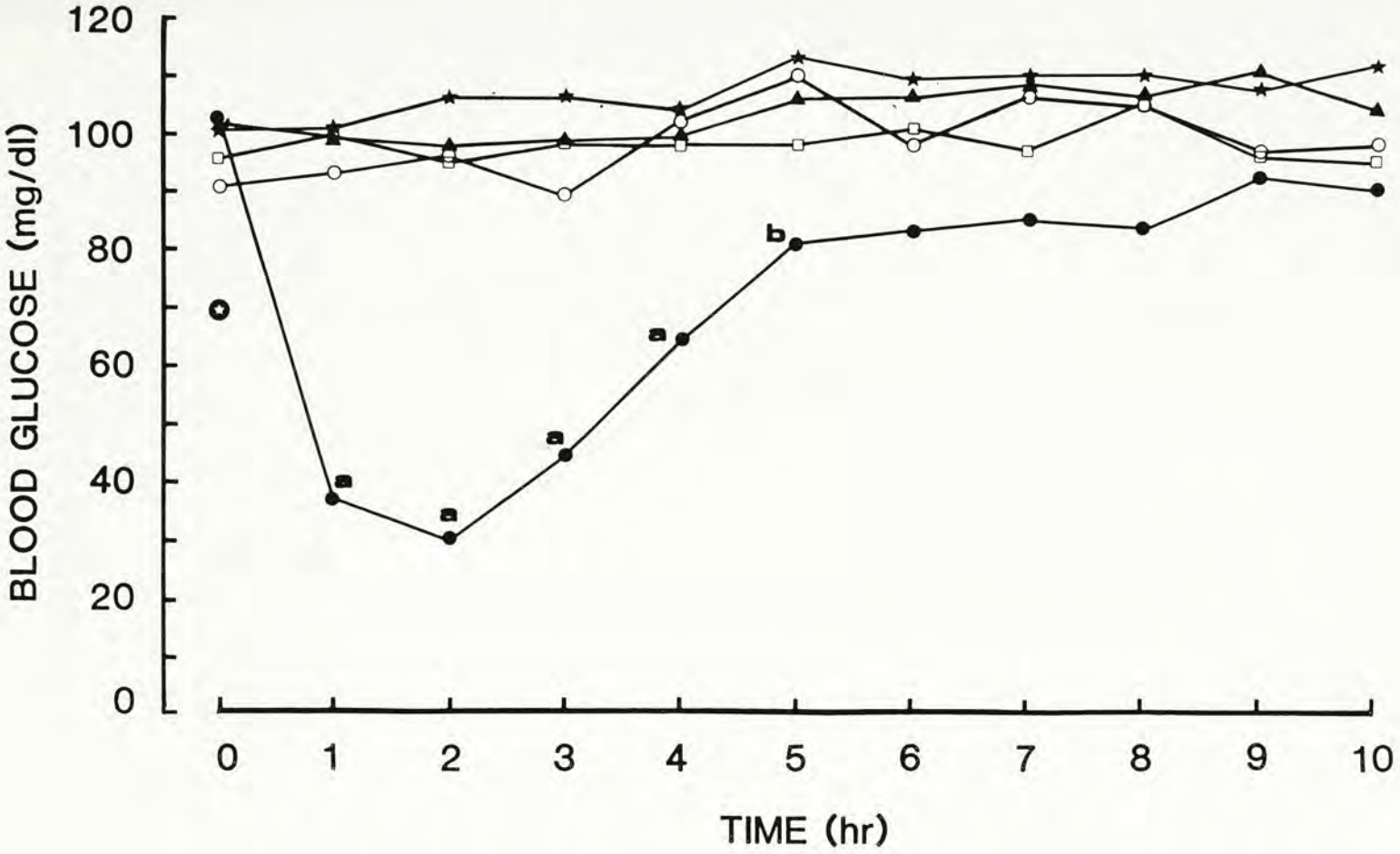


Figure 2-34 Blood glucose levels of fasting old rats at various time intervals after subcutaneous administration of saline (as control) (★), 1 U/Kg insulin (26.5 U/mg) (●), 2 mg/Kg C_{6-1} (▲), 5 mg/Kg C_{6-1} (○), and 2 mg/Kg C_{1-3} (□). The blood glucose concentration of old rats was higher than that of the young rat (✪). Insulin greatly decreased blood glucose whereas C_{1-3} and C_{6-1} were ineffective when tested at the above doses. Number of animals in control, 2 mg/Kg C_{6-1} treated, 5 mg/Kg treated C_{6-1} treated, C_{1-3} treated and insulin treated groups were 7, 7, 3, 6 and 6 respectively.

a = Highly significant decrease as compared to the preinjection vale ($p < 0.001$)

b = Significant decrease as compared to the prejection value ($p < 0.05$)

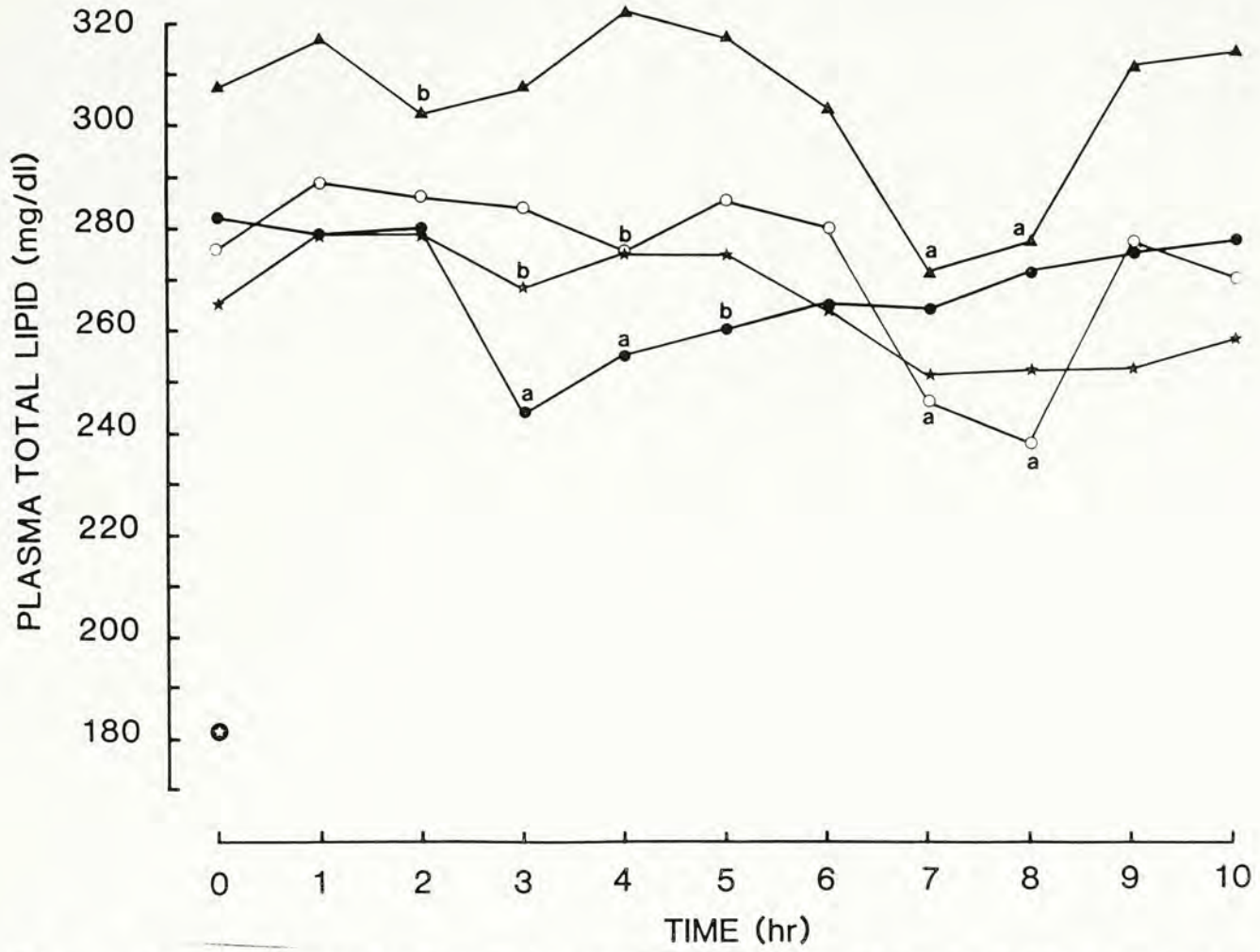


Figure 2-35 Plasma total lipid levels of fasting old rats at various time intervals after subcutaneous administration of saline (as control)(★) , 2 mg/Kg C_{1-3} (○), 2 mg/Kg C_{6-1} (▲) and 1 U/Kg insulin (26.5 U/mg) (●). The plasma total lipid level of old rats was higher than that of the young rats (✪). Number of animals in control, C_{6-1} treated , C_{1-3} treated and insulin treated groups were 7, 7, 6 and 6 respectively.

a = Significant decrease as compared to the preinjection value ($p < 0.05$)

b = Not statistically significant when compared with the preinjection value ($p > 0.05$)

would still be inappropriate to draw any conclusion on the effect of both proteins on total lipid level because the control group showed a similar fall at that time intervals.

Since the molecular weights of both C 1-3 and C 6-1 have not yet been determined, their relative hypoglycemic and *in vitro* lipogenic potencies were compared with those of insulin on a weight basis. Insulin (26.5 U/mg) injected at a dose of 37.7 µg/Kg or 1 U/Kg caused a drastic decrease in blood glucose while a 2 mg/Kg dose of C 1-3 and 5 mg/Kg dose of C 6-1 did not exhibit any significant hypoglycemic effect. The lipogenic activity of 37.7 ng/ml or 1 mU/ml insulin was comparable to those of 200 µg/ml of both poteins. The great difference in potency among them may be attributed to the fact that insulin is an endogenous hormone while both proteins are exogenous compounds purified from plant.

3. ABORTIFACIENT PROTEIN, SAPONINS AND LECTINS FROM OTHER PLANTS

3. ABORTIFACIENT PROTEIN, SAPONINS AND LECTIN FROM OTHER PLANTS

3.1 Abortifacient protein and lectin of Tianhuafen

3.1.1 Introduction

The Chinese drug Radix Trichosanthis is the root tuber of the medicinal plant Trichosanthes kirilowii Maxim, Cucurbitaceae. Since the powdered root is as white as snow, Radix Trichosanthis gains the name Tianhuafen (the powder of heavenly flower) (Li, 1596).

3.1.1a Effect of Tianhuafen on blood glucose level

Tianhuafen is one of the most frequently used diabetic remedies in traditional Chinese herbal medicine. For instance, in the book "One Thousand Golden Prescriptions", out of the 52 prescriptions for curing diabetes, there are 23 containing Tianhuafen. The intention of using these drugs is to "produce the sap" and "clean the heat" or "cool the system" (Chen 1981). However, it was found that Tianhuafen (both infusion and decoction) produced rapid hyperglycemia in normal rabbits 0.5 - 1 hour after oral administration (Puingki, 1930). An ethanolic extract of Tianhuafen administered at a dose of 5 g/kg body weight did not produce hypoglycemic effect in normal and alloxan-induced diabetic rabbit (Anon., 1977).

3.1.1b Abortifacient activity

In "Pen Ts'ao Kang Mu" (The Great Pharmacopoeia), Tianhuafen was described to have the function of "inducing menstrual fluid" and to "expel fetal membranes" (Li, 1596). It has been traditionally applied externally as an abortifacient

drug of mid-gestation (Anon., 1976). The active component, a basic protein, named α-trichosanthin, with a molecular weight of approximately 24,000, was first isolated by Wang et al. (1976). This protein is very effective, both in humans and in laboratory animals, in inducing mid-term abortion by selectively causing the necrotic denaturation of the syncytiotrophoblasts of placental villi (Lau et al., 1981). It is also effective in curing ectopic pregnancy, hydatiform mole, and invasive mole and it has some therapeutic action on choricarcinoma (Anon., 1976).

Although both momorcharins isolated from M. charantia seed possessed no intrinsic lipolytic, antilipolytic or lipogenic activity, incubation with corticotropin for several hours at room temperature resulted in an impairment of the lipolytic activity of the hormone. As we know that during fetal life, corticotropin controls the human adrenal which is large and actively secretes sulfate conjugates of androgens that are converted in the placenta to androgens and oestrogens which enter the maternal circulation for the maintenance of pregnancy (Ganong, 1981). The possibility of the impairment of the biological activity of corticotropin as one of the mechanisms of both momorcharins and other abortifacient proteins could not be ruled out. So the effect of trichosanthin, a mid-term abortifacient protein from Tianhuafen on lipid metabolism in isolated rat adipocytes was examined.

3.1.1c Lectins

A galactose binding lectin from Tianhuafen was isolated

by Yeung et al. (1980). The lectin did not inhibit the binding of ^{125}I-insulin to its receptor at high concentration but at low concentration, it showed slight stimulation (Feng et al., 1981).

In view of the strong antilipolytic and lipogenic activities of M. charantia lectin, Tianhuafen lectin was also studied to see whether it possessed insulin-like activity. Their chromatographically related fractions were also examined to see if the purification method could result in the isolation of insulin-like compound(s) of non-lectin nature that could account for Tianhuafen as folkloric diabetes remedies.

3.1.2 Materials

Fresh root tubers of Trichosanthes kirilowii obtained from Kwongsi, China were used as Tianhuafen for this study. The suppliers of all the chemicals and apparatus used in lipolysis and lipogenesis assays were listed in chapter one.

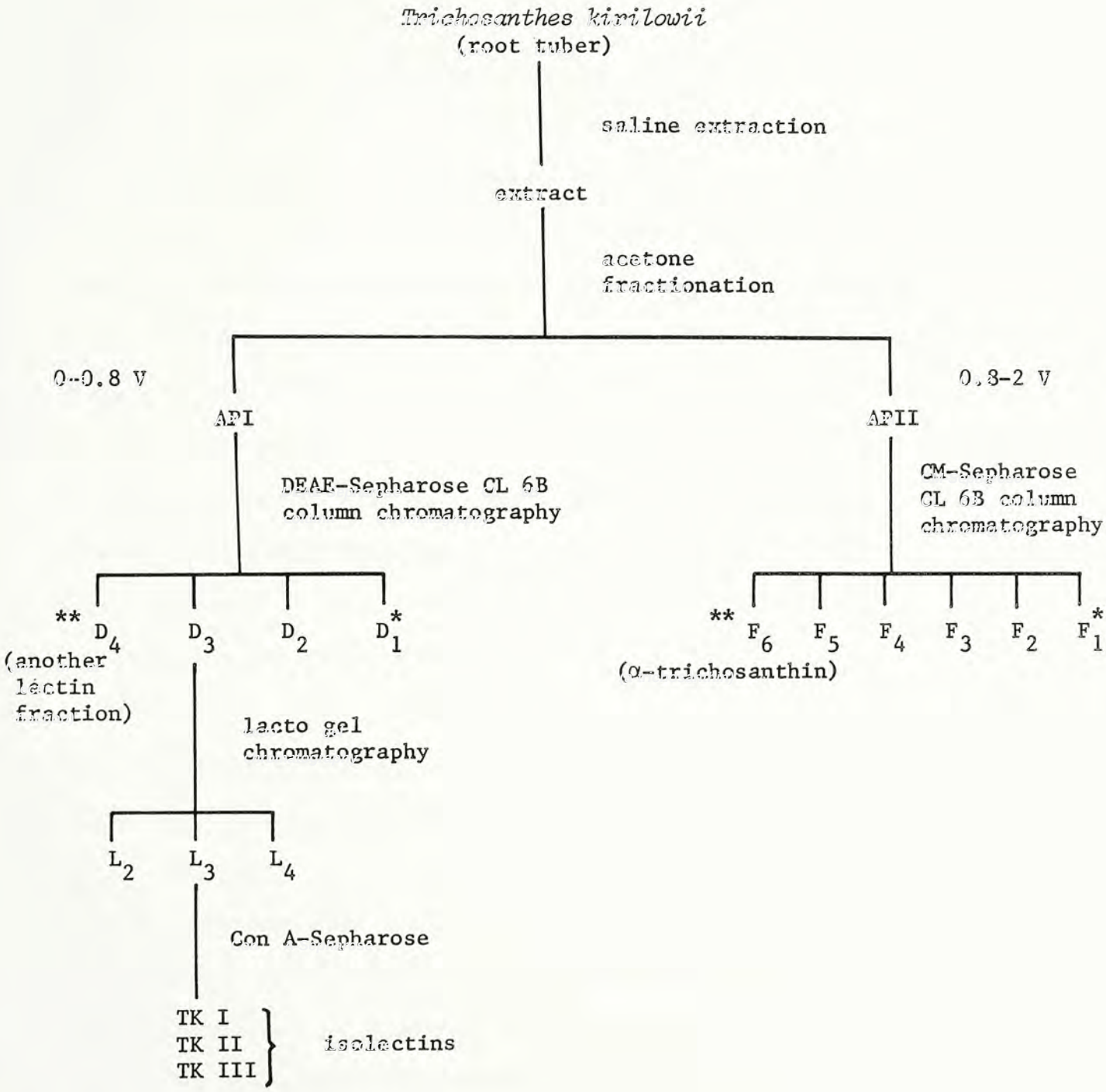
3.1.3 Methods

All the fractionation and purification work were done in our laboratory by Miss W.W. Li and Mr. K.C. Pong during the whole study. Chart 6 summarized the purification steps of the abortifacient protein, α-trichosanthin (F_6), and a galactose binding Trichosanthes kirilowii lectin.

The major abortifacient protein is F_6, but F_3, F_4 and F_6 are chromatographically related fraction, which show similar abortifacient activities, molecular weights and amino acid compositions and identical immunoelectrophoretic behavior. They

Chart 6

Purification steps of an abortifacient protein and a galactose-binding lectin from *Trichosanthes kirilowii* (Tianhaufen).



* : unretarded fraction

** : most retarted fraction

differ in charge properties on account of varying degrees of adsorption on CM-Sepharose CL-6B (Wong, 1979).

The Trichosanthes kirilowii lectin preparation tested for antilipolytic and lipogenic activities were designated D_3 and D_4. They were obtained by fractionating a saline extract of the roots with acetone and subsequently chromatographing the fraction on DEAE-Sepharose CL-4B (Pharmacia) and eluting the adsorbed material with a concentration gradient of NaCl. Three isolectins could be isolated from D_3 by affinity chromatography on lacto-gel (E.Y. Laboraties) and then on Con A - Sepharose (Pharmacia) (Wong, et al., 1982). D_4 was a fraction more strongly adsorbed on DEAE-Sepharose than D_3. It showed some common bands with D_3 in electrophoresis and had potent hemagglutinating activity. However, preliminary experiments showed that lectins could not be purified from D_4 by affinity chromatography with the same ease as D_3 (data from our laboratory).

3.1.4 Results and discussion

3.1.4a Abortifacient protein and its chromatographically related fractions

Trichosanthin (F_6) and chromatographically related fractions, F_1 to F_5, were devoid of any major lipolytic activity (Table 3-1) and antilipolytic activity (Table 3-2) at dose tested. Trichosanthin, like α-momorcharin and β-momorcharin, did not exert any effect on the incorporation of tritiated glucose into lipid (Table 3-3).

3.1.4b Lectin and its chromatographically related fractions

Table 3-1

Assay of F_6 and its chromatographically related fractions for lipolytic activity

Expt. No.	Fraction	Dose (µg/tube)	Glycerol production (µmol/hr/g fat cell dry wt)
1	None	-	1.60 ± 0.06
	F_6	100	1.43 ± 0.03
2	None	-	2.05 ± 0.07
	$F_1 + F_2$	100	1.93 ± 0.05
	F_3	100	1.67 ± 0.004
	F_4	100	2.01 ± 0.07
	F_5	100	2.04 ± 0.05

Table 3-2

Effects of F_6 and chromatographically related fractions in inhibiting corticotropin- and epinephrine- induced lipolysis

Antagonizing Fraction	Dose (μg/tube)	% of 25 mU ACTH-induced lipolysis	% of 0.33 μg epinephrine bitartrate-induced lipolysis
None	-	100	100
Propranolol	100	24.2 ± 1.0	
	33	67.5 ± 0.8	-
	11	91.7 ± 3.4	
F_1	250	101.2 ± 1.3	107.3 ± 5.2
F_2	250	104.6 ± 1.0	117.9 ± 1.8
F_3	250	98.3 ± 1.7	115.9 ± 1.3
F_4	250	107.4 ± 2.9	111.2 ± 1.7
F_5	250	115.7 ± 2.3	113.8 ± 3.8
F_6	250	122.1 ± 5.1	106.8 ± 3.7

ACTH (25 mU) elicited a net increase of glycerol production over the control at a rate of 4.29 ± 0.08 μmol glycerol per hr per g fat cell dry weight. The corresponding value for epinephrine (0.33 μg)-induced lipolysis was 2.86 ± 0.04.

Table 3-3

Lipogenic activities of F_1, F_2 and F_6

Fraction	Dose	D-[3-3H]-glucose incorporated into lipid (cpm)
Control	-	2066 ± 163
Insulin	1000 μU	6253 ± 84
	10 μU	3532 ± 199
F_1	100 μg	2048 ± 94
	20 μg	1744 ± 5
F_2	100 μg	2697 ± 548
	20 μg	1891 ± 55
F_6	100 μg	2085 ± 278
	20 μg	1964 ± 30

D-[3-3H]-Glucose (84130 ± 842 cpm), and around 2×10^4 fat cells (3.56 mg total lipid) were added to each assay tube.

Table 3-4 presents the lipogenic activities of D_3 and D_4. The lectin preparations lacked intrinsic lipolytic activity (Table 3-5) and any inhibitory effect on epinephrine- and corticotropin-induced lipolysis (Table 3-6). A similar lack of antilipolytic activity was observed in isolated hamster adipocytes (Table 3-7). In both the rat adipocytes (Table 3-6) and hamster adipocytes (Table 3-7) the lectin preparations appeared to enhance the lipolytic effect of corticotropin and epinephrine.

Although the galactose binding lectin from T. kirilowii elicited lipogenic activity in isolated rat epididymal adipocytes, it differed from insulin and other lectins in that it did not antagonize hormone-induced lipolysis even when tested at a dose as high as 1000 µg in rat adipocytes and 300 µg in hamster adipocytes. It appears that the lectin did not act either as a β-adrenergic antagonist or as an α-adrenergic agonist because it was devoid of any antilipolytic activity in rat adipocytes which possessed only β-adrenoceptors as well as in hamster adipocytes which also possessed α-adrenoceptors (Carpere, et al., 1983). Neither did it interact with the corticotropin receptors in hamster adipocytes to bring about inhibition of lipolysis.

Katzen et al.(1981) and Hedo et al. (1981) showed that galactose binding lectin with galactose residues in the β configuration, such as ricin II, manifested potent antilipolytic activity and markedly enhanced glucose oxidation in adipocytes while other galactose binding lectins were much less active, presumably because their galactose residues do not possess the

Table 3-4

Lipogenic activities of D_3 and D_4

Expt. No.	Fraction	Dose	% of added counts/10 mg lipid
1	None	-	0
	D_3	500 µg	5.60 ± 0.27
		100 µg	3.41 ± 0.97
	D_4	500 µg	6.48 ± 0.42
		100 µg	3.72 ± 0.23
2	None	-	0
	Insulin	1 mU	13.99 ± 0.28
		10 µU	4.89 ± 0.67
	D_3	100 µg	3.25 ± 0.70
		20 µg	2.90 ± 1.25
	D_4	100 µg	2.90 ± 0.44
		20 µg	0.79 ± 0.56
3	None	-	0
	Insulin	1 mU	27.82 ± 0.08
	Concanavalin A	100 µg	27.46 ± 0.73
	D_3	60 µg	1.92 ± 0.22
	D_4	60 µg	1.66 ± 0.31

79130 ± 365, 84130 ± 842 and 94038 ± 384 cpm D-[3-3H]-glucose and fat cells containing 3.05, 3.56 and 5.44 mg lipid were added to each vial in experiment 1, 2 and 3 respectively.

Table 3-5

Assay of D_3 for lipolytic activity

Fraction assayed	Dose	Glycerol production µmol/hr/g fat cell dry wt
None (control)	—	1.32 ± 0.05
D_3	100 µg	1.36 ± 0.02 (NS)
ACTH	25 mU	4.76 ± 0.12 ($p < 0.001$)

NS = Statistically not significant compared with control.

Table 3-6

Effect of D_3 and D_4 on epinephrine- and corticotropin-induced lipolysis in rat adipocytes

Expt. No.	Antagonistic fraction	Dose (μg/tube)	% of epinephrine (0.33 g)-induced lipolysis	% of ACTH (25mU)-induced lipolysis
1	None (control)	—	100	—
	Concanavalin A	100	45.5 ± 1.0	—
		20	50.5 ± 0.0	
		40	63.1 ± 0.6	
	D_3	50	128.0 ± 3.1 ($p < 0.01$)[a]	—
		10	117.9 ± 1.0 ($p < 0.1$)	
		2	112.0 ± 1.2 ($p < 0.1$)	
	D_4	50	123.1 ± 4.4 ($p < 0.01$)	—
		10	110.5 ± 3.3 ($p < 0.1$)	
		2	100.5 ± 1.3	
2	None (control)	—	100	100
	D_3	1000	177.4 ± 2.6($p < 0.005$)[a]	120.6 ± 2.9($p < 0.01$)

Epinephrine (0.33 μg) and ACTH (25 mU) elicited an increase of glycerol production over the control at the rate of 14.05 ± 0.14 and 4.29 ± 0.08 μmol glycerol production per hr per g fat cell dry weight respectively.

a : Compared with control.

Table 3-7

Effect of D_3 and D_4 on corticotropin-induced lipolysis in hamster

Antagonistic fraction	Dose (μg/tube)	% of ACTH (0.5 mU)-induced lipolysis
None	—	100
Concanavalin A	300	0
	100	57.06 ± 3.04
D_3	300	198.88 ±16.82
D_4	300	529.64 ±19.72

requisite configuration. The lack of any significant antilipolytic activity in T. kirilowii lectin and its possession of only a slight lipogenic activity suggest that the lectin possessed an α-galactose residue. Because the lectin exhibited some but not all of the insulin-like activities, it would be of great interest in a future study to see if it can bind to insulin receptors.

It is noteworthy that the T. kirilowii lectin, at the dose of ca. 100 µg slightly potentiated the lipolytic actions of corticotropin and epinephrine in rat adipocytes while it did not possess intrinsic lipolytic activity. Further studies would be required to elucidate the mechanism of such potentiating effect. The lipolytic activity of the lectin seen at a dose of 1000 µg in rat adipocytes and 300 µg in hamster adipocytes is probably due to the massive doses used.

The present results add new information to the existing literature on the insulinomimetic actions of lectins. Previous reports e.g. that of Katzen et al.(1981) did not look for a possible lipogenic effect of those lectins that manifest minimal or no antilipolytic activity. The T. kirilowii lectin furnishes an example of a lectin that is deficient in antilipolytic activity but potent in stimulating lipogenesis from glucose. It is also capable of potentiating the lipolytic action of epinephrine and corticotropin.

More about the relationship between carbohydrate binding specificities of other lectins and their abilities to bind hormone receptors will be discussed later.

3.2 Ginseng saponins (Ginsenosides)

3.2.1 Introduction

The ginseng plant belongs to the Araliaceae family. There are two separate sources for the commercial product of ginseng. Panax ginseng C.A. Meyer, is the source of the Chinese, Korean and Japanese ginseng; while Panax quinquefolius L. is the source of American ginseng (Hu, 1977).

3.2.1a Ginseng saponins

The dried roots of ginseng contain many physiologically active constituents among which the saponins, also called panaxosides and ginsenosides, are the major components and have been extensively studied.

In 1964, Shibata and his co-workers (Sanada et al., 1974a; 1974b) isolated 13 ginsenosides from ginseng root extract. They are : Ro, Ra, Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_2, Rg_3, Rh_1 and Rh_2. These ginsenosides can be divided into three groups depending on the aglycone portion of the molecules. Ginsenoside Ro gives oleanolic acid, while ginsenosides Ra, Rb, Rc, Rd give panaxadiol and ginsenosides Re, Rf, Rg, and Rh give panaxatriol after acid hydrolysis. Oleanolic acid is a pentacyclic, oleanane-type triterpene compound. Ginsenosides of the Rb and Rg groups are tetracyclic dammarane-type triterpenes (Sanada et al., 1974a; 1074b).

3.2.1b Effects of ginseng on sugar and lipid metabolism

From ancient times, ginseng has been used for the treatment of various diseases including diabetes mellitus.

Petkov (1961) reported that oral administration of an aqueous alcoholic extract of ginseng root caused a decrease in the blood glucose concentration of rabbits. Ginseng saponins were found to be able to reduce adrenaline-induced hyperglycemia (Takeuchi, 1965; Yokozawa et al., 1975; Yokozawa and Oura, 1976). The pathological condition of diabetic patients could be improved by administering red ginseng (Okuda et al., 1980). When ginseng saponins (30 mg/kg) were administered 7 hours before adrenaline administration, plasma concentrations of glucose, triglycerides and fatty acids as well as lipase activity significantly decreased in a dose dependent manner when compared with control. Apparently with the controls, the protopanaxatriol group has a potentiating effect on adrenaline-induced hyperglycemia, but the protopanaxadiol group has an inhibitory effect. In addition, ginseng saponins seem to have a strong inhibitory action on triglyceride mobilization into blood and a stimulatory effect on esterification of free fatty acids in liver and adipose tissue (Rhee et al., 1981). Ginseng stimulated the biosynthesis of cholesterol in liver in vivo (Sakakibara et al., 1975) but not in liver slices in vitro (Gommori et al., 1976). In normal rats, serum cholesterol level decreased in proportion to the dose of purified total saponin. In rats treated with a high dose of cholesterol, oral administration of 150 mg total saponin per Kg body weight decreased the serum cholesterol level (Lim et al., 1981). Ohminami et al. (1981) found that ginseng saponins did not affect adrenaline-induced lipolysis in isolated rat adipocytes but corticotropin-induced lipolysis was inhibited by ginsenoside Rbc group, Rb_1, Rb_2, Rc, Rg group, Re, Rg_1 and Rg_2.

Ginsenoside Rbc group, Rb_2, Rc, Rg group, Rg_1, Rg_2 and Rh_1 suppressed insulin-stimulated lipogenesis in adipose tissue slices while ginsenoside Rb_1, and Re did not inhibit lipogenesis.

Kimura et al. (1981a) demonstrated that DPG-3-2, a partially purified Panax ginseng fraction, lowered the blood glucose level and stimulated insulin release in diabetic animals. In addition it stimulated insulin biosynthesis in different preparations of pancreas from hyperglycemic animal. DPG-3-2 consisted of unknown substances as major components and ginseng saponin (Ginsenoside Rg_1) as minor component. However purified ginsenoside Rg_1 did not lower the blood glucose level. Hence the hypoglycemic effect of DPG-3-2 did not appear to be related to the saponins present as minor components in the fraction. The hypoglycemic component must therefore have been a principle distinct from saponins. This hypoglycemic fraction did not antagonize epinephrine-induced lipolysis but stimulated glucose utilization. It enhanced glucose-induced insulin release from pancreatic islets and thereby lowered the blood glucose level (Kimura et al., 1980b; Waki et al., 1982). It increased rat hepatic glycogen content, decreased the blood level of ketone bodies in alloxan-induced diabetic mice, and inhibited the release of free fatty acids from rat epididymal fat pads (Kimura et al., 1981a). This shows that the saponins are not the only components responsible for the pharmacological action of ginseng radix especially with respect to its hypoglycemic action.

The aim of the present study was to further investigate whether the antilipolytic and antilipogenic effects of ginseng

saponins (ginsenosides), Rg_1, Rg_2, Rc, are due to the decrease in cell viability or interaction with the hormone receptor on adipocytes.

3.2.1c Insulin-like compounds

A heat stable acidic peptide, composed of 14 amino acids (molecular weight 1,400) and exhibiting insulin-like activity, was isolated from Panax ginseng radix (Ando et al., 1980). Okuda et al. (1980) found that adenosine in ginseng exhibited insulin-like activity. Both the peptide and adenosine suppressed corticotropin-, adrenaline-, and growth hormone-induced lipolysis while stimulating lipogenesis from glucose in fat cells (Okuda et al., 1980). Sekiya et al. (1981) purified another antilipolytic substance from Panax ginseng. This crystallizable active substance is a carboxylic acid. It was found to inhibit exclusively catecholamine-induced lipolysis, while it failed to inhibit lipolysis induced by peptide and protein hormones such as corticotropin, glucagon and thyroid stimulating hormone.

3.2.2 Materials and methods

Ginsenoside Rg_1, Rb_2, and Rc were purified according to (Chen et al., 1978) in our laboratory. All the materials and methods used in lipolysis and lipogenesis assays were the same as described in chapter one.

3.2.3 Results and Discussion

Increasing the dose of corticotropin could not overcome

the inhibitory effect of ginsenoside Rc, Rb$_2$ and Rg$_1$, on rat adipocyte lipolysis stimulated by the hormone (Figure 3-1) i.e. non-competitive inhibitors of corticotropin-induced lipolysis. The suppression of dbcAMP-induced lipolysis by the three ginsenosides could not be reversed by increasing the doses of the lipolytic stimulus (Figure 3-2). This suggests that they act at a locus on the adipocytes beyond the generation of dbcAMP.

The three ginsenosides did not affect the basal glucose incorporation into total lipids (Table 3-8). Ginsenoside Rg$_1$ and Rc inhibited insulin (1 mU) stimulated lipogenesis. In contrast to the finding of Ohminami et al. (1981), ginsenoside Rb$_2$ did not suppress insulin's lipogenic effect on adipocytes (Table 3-9). This shows that the antilipogenic effect of ginsenoside Rg$_1$ and Rc is not due to the inhibition of glucose uptake but to the inhibition of the stimulated lipogenesis by insulin.

The parallel increase in glycerol release with the increasing doses of corticotropin and dbcAMP and the unaffected basal glucose incorporation into total lipid indicated that the antilipolytic effect of the three ginsenosides and the antilipogenic effect of ginsenoside Rg$_1$ and Rc were not due to a decrease in cell viability. The antilipogenic effect of ginsenoside Rg$_1$ and Rc was probably due to an inhibition of the insulin-stimulated glucose transport into the adipocytes.

3.3 Other lectins

3.3.1 Introduction

Lectins (from the Latin legere : to choose or pick)

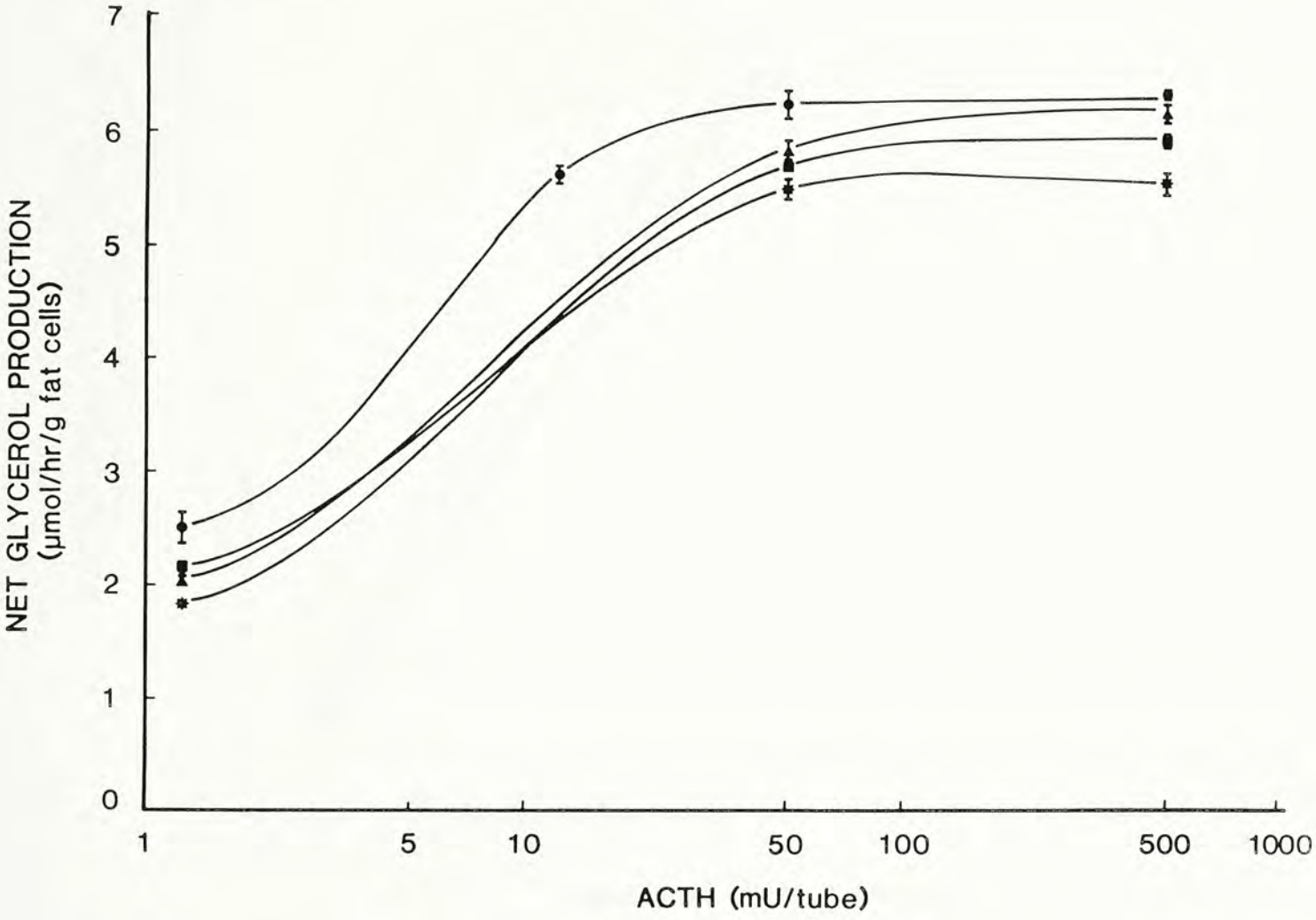


Figure 3-1. Effect of ginseng saponins on lipolysis induced by varying doses of ACTH (●). The inhibitory effects of 100 ug ginsenoside Rc (▲), Rb_2 (■) and Rg_1 (✸) could not be overcome by increasing the dose of the hormone.

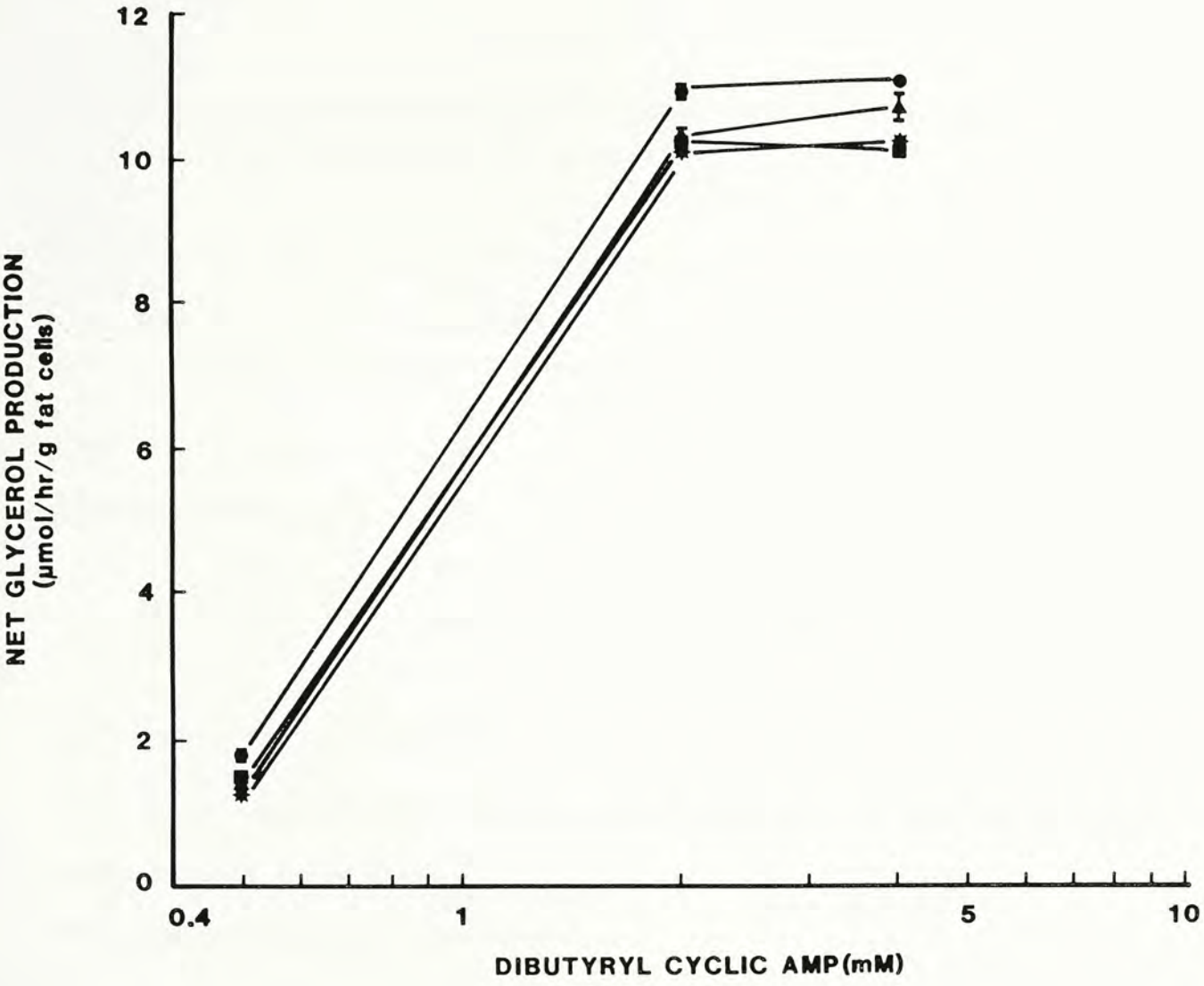


Figure 3-2. Effect of ginseng saponins on lipolysis induced by varying doses of dbcAMP (●). The inhibitory effect of 100 µg ginsenoside R_{g1} (■), R_{b2} (▲) and R_c (✱) could not be overcome by increasing the dose of dbcAMP.

Table 3-8

Effect of ginsenosides on basal glucose incorporation into total lipids in isolated rat adipocytes

Fraction assayed	Dose (μg/tube)	D-[3-3H]-glucose incorporates into lipid (cpm)
None	-	8533 ± 314
Ginsenoside Rg_1	300	7905 ± 177 (NS)
Rb_2	300	8341 ± 154 (NS)
Rc	300	9099 ± 100 (NS)

D-[3-3H]-Glucose (98256 ± 660 cpm) and about 1.3×10^5 adipocytes were added to each assay vial.

NS = Statistically not significant.

Table 3-9

Effect of ginseng saponins on insulin-stimulated lipogenesis from glucose in isolated rat adipocytes.

Addition	Dose	% of added counts/10 mg lipid
None	-	0
Insulin	1 mU	21.09 ± 0.98
Insulin + ginsenoside Rg_1	300 µg	16.66 ± 0.22($p < 0.0025$)[a]
Insulin + ginsenoside Rb_2	300 µg	20.06 ± 0.39(NS)[b]
Insulin + ginsenoside Rc	300 µg	18.89 ± 0.13($p < 0.005$)[a]

D-[3-3H]-Glucose (98256 ± 660 cpm) and fat cells containing 8.26 mg lipid were added to each assay vial.

a : Compared with addition of insulin alone.

b : Statistically not significant compared with addition of insulin alone.

(Boyd et al., 1954) are a group of proteins or glycoproteins of non-immune origin which bind more or less specifically to particular carbohydrate molecules and are capable of agglutinating cells / or precipitating glycoconjugates. They are also known as hemagglutinins, phytohemagglutinins, agglutinins and protectins because of their hemagglutinating activity and plant origin (Sharon, 1977). Other cell types including lymphocytes, fibroblasts, sperms, bacteria and fungi are also agglutinated by lectins. Lectins are mainly present in the seeds of plants though there are reports of lectins in leaves, stems and roots of some plants (Horejsi et al., 1978; Goldstein et al., 1978). Some were isolated from bacteria, slime molds and algae and some from invertebrates such as snails and horseshoe crabs (Barondes, 1981). In recent years, it has been found that electric eels, chicken heart, rabbit liver, mouse thymus, bovine spleen and even human liver also contain lectins (Sun et al., 1981). To date over 100 different lectins have been purified and chemically characterized, most of them from plants. They differ widely in their physical and chemical properties such as molecular weight, carbohydrate content, number of subunits as well as number of sugar-binding sites. The molecular weights of lectins range from 12,000 for Agardhiella tenere agglutinin to 400,000 for horseshoe crab lectin.

According to the definition of Goldstein et al. (1980), lectins must bear at least two sugar-binding sites, agglutinate animal cells and plant cells, and / or precipitate polysaccharide, glycoproteins and glycolipids. Strictly speaking, toxins like ricin, abrin and modeccin etc., which bear

only one sugar-binding sites, are not regarded as lectins since they do not agglutinate cells or precipitate glycoconjugates.

3.3.1a Classification

The specificity of a lectin is usually defined in terms of the monosaccharides or simple oligosaccharides needed to inhibit lectin-induced agglutination or precipitation reactions (Goldstein et al., 1980). According their sugar specificity, lectins are now commonly classified into several groups : α-D-glucose binding, α-D-mannose binding, β-D-galactose binding, α-L-fucose binding, N-acetylneuraminic acid binding, N-acetylglucosamine binding, N-acetylgalactosamine binding, (β-(1→4)-D-glucosamine N-acetyl) binding and D-galactose-β(1→3)-galactosamine N-acetyl binding (Goldstein, 1981). Nevertheless, it is noteworthy that some D-glucose-binding lectins bind N-acetyl-D-glucosamine as tightly as D-glucose and may be placed in either D-glucose-binding or N-acetyl-D-glucosamine-binding group. The same applied to some D-galactose-binding lectins. In addition, many lectin, differ markedly with respect to their anomeric specificity to monosaccharides (Goldstein et al., 1978).

3.3.1b Insulin-like activity

Apart from probable endogenous biological functions such as cellular recognition (Barondes, 1981), many other biological properties such as stimulation of mitosis and blastogenic transformation of lymphocytes, preferential agglutination of malignant cells, and toxicity toward different

cell lines (Sharon, 1977) and insulin-like activity have been described for lectins.

Concanavalin A (Con A), wheat germ agglutinin and several other plant lectins that are commonly used as probes for identifying and mapping cell surface sugars and studying cell surface architecture exhibit a variety of insulin-like activities *in vitro*. Con A and wheat germ agglutinin are as effective as insulin in stimulating glucose oxidation and hexose transport in isolated fat cells. Both lectins inhibit epinephrine-induced lipolysis in isolated fat cells, adenylate cyclase activity of

fat cell membrane and I-insulin binding to intact adipocytes and liver cell membranes (Czech *et al.*, 1973; Cuatrecasas *et al.*, 1973; Cuatrecasas, 1973; Czech, 1974; Livingston *et al.*, 1980). Cuatrecasas & Tell (1973) successfully used Con A-Sepharose affinity chromatography to extract insulin receptor from detergent-solubilized liver cell membranes.

Cuatrecasas & Tell (1973) speculated that Con A might exert its insulin-like activity by interacting directly with glycoprotein insulin receptors and even sharing as structural similarity in their binding regions. On the other hand, Czech *et al.* (1974) and Carter-Su *et al.* (1980), concluded that, because bioactivities of insulin and insulin binding to fat cells were sensitive to trypsin treatment of the cells while those properties of Con A were not, the lectin's activities were probably mediated by receptor moieties distinct from those involved in insulin's action. Katzen *et al.* (1981) presented evidence supporting the concept that the multiple insulin-like activities of Con A were mediated by the same effector system as

that responsible for insulin's actions. For instance, the ratio of Con A's to insulin's half-maximal effective concentrations required for effects on glucose oxidation, lipogenesis and glucose uptake was essentially constant. This suggests a close linkage between Con A's effector system and the insulin receptor. In addition, the effects of Con A were additive to those of insulin. Con A, like insulin, stimulated diaphragm muscle glycogenesis and adipose tissue lipogenesis *in vivo*. Hedo *et al.* (1981) using sequential lectin chromatography discovered the carbohydrate moiety of the insulin receptor contains N-acetylglucosamine, mannose and galactose as common determinants but that these saccharide residues are neither receptor nor tissue specific. Shechter (1983) showed that these externally located fat cell surface determinants, which, upon being occupied continuously produce persistant insulin-like activities. Those lectins with insulin-like activity may be useful tools in better understanding of the mechanism of action of insulin as well as the structure of insulin receptor.

3.3.1c Carbohydrate binding specificity and insulin receptor binding

The relationship between the carbohydrate binding specificity of a lectin and its ability to bind insulin receptors and evoke insulin-like effects in isolated rat adipocytes has been examined by Katzen *et al.* (1981) and Hedo *et al.* (1981). They have demonstrated that mannose binding lectins including Concanavalin A, garden pea lectin and lentil lectin, and the N-acetylglucosamine binding lectin wheat germ lectin, bind to

insulin receptors and produce insulin-like activities in isolated rat adipocytes such as antilipolysis and enhancement of glucose oxidation. Insulin receptor nonbinders, including the fucose specific lectin from gorse seeds and galactose binding lectins from peanut, Bandieraea simplicifolia and Tianhuafen discussed in section 3.1.4b fail to evoke such responses from the adipocytes. However, in contrast to these results, ricin II, also a galactose binding lectin, exerts insulinomimetic-activities (Katzen et al., 1981). Similarly a galactose binding lectin from the seeds of the bitter gourd Momordica charantia, discussed in the preceding Chapter, manifests potent antilipolytic and lipogenic activities. As mentioned before, Hedo et al. (1981) attributes such discrepancy among the galactose binding lectins to differences in the configuration of the galactose residue which is in the insulin receptor nonbinders and in the binders.

The intent of the present study was to extend the work of Katzen et al. (1981) by examining more plant lectins and comparing the potencies of the various lectins in inhibiting hormone-induced lipolysis and augmenting lipogenesis. In studying the antilipolytic effects of lectins we have selected, besides the commonly employed rat adipocyte system, the hamster adipocyte system. While the rat adipocyte lacks functional α-adrenergic receptors the hamster adipocyte possesses both α- and β-adrenergic receptors (Carpere et al., 1983). We have also attempted, on account of molecular weight differences among lectins, to compare the relative antilipolytic potencies of the various lectins on a molar basis instead of the weight basis

utilized by Katzen et al. (1983). Furthermore we utilized lipogenesis as an assay parameter of insulin-like activities instead of stimulation of glucose oxidation (Katzen et al., 1981) because the latter parameter is also affected by hormones like epinephrine and corticotropin (Rodbell, 1964) and is therefore not an activity specific to insulin. As mentioned above, although extensive research has been done on the mechanism of the interaction of Con A and wheat germ agglutinin with the insulin receptor, little is known about their interaction with the corticotropin and epinephrine receptors on rat adipocytes. Here, a preliminary investigation of their binding capacity to hormone receptors was also included.

3.3.2 Materials and Methods

Lectins from garden pea (Pisum sativum), Concanavalin A, lentil (Lens culinaris), wheat germ (Triticum vulgaris), gorse (Ulex europeus), and asparagus pea (Tetragonolobus purpureas) were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Partially purified potato lectin was obtained from PL Biochemicals. Lectins from Bauhinia purpureas, Bandeiraea simplicifolia II, Cytisus sessilifolius, horse gram (Dilichos biflorus), lima bean (Phaseolus limensis), osage orange (Maclura pomifera), peanut (Arachis hypogaea), soybean (Glycine max), Sophora japonica and Wisteria floribunda were purchased from E.Y. Lab. Momordica charantia lectin was isolated from seeds in our laboratory.

Lipolysis and lipogensis assays were carried out as described in chapter one.

3.3.3 Results and Discussion

The molecular weight and carbohydrate specificity of the lectins that we examined are listed in Table 3-10. Table 3-11 presents the relative antilipolytic activities of the various lectins. The Momordica charantia lectin, a galactose binding lectin, and the highest antilipolytic activity. The mannose binding lectins including Concanavalin A, garden pea lectin and lentil lectin all had comparable activity and they belong to the group with the second highest activity. The N-acetylglucosamine binding wheat germ lectin and the N-acetylgalactosamine binding Wisteria floribunda lectin had lower activity than the mannose binding group. However, the fucose binding asparagus pea lectin was devoid of any significant antilipolytic activity, and so were the galactose binding Bandeiraea simplicifolia and Cytisus sessilifolius and the N-acetylgalactosamine binding Bauhinia purpureas, Phaseolus limensis and Maclura pomifera lectins (Table 3-12).

The mannose binding lectins had the highest lipogenic activity, followed by wheat germ lectin. M. charantia, Wisteria floribunda and osage orange lectins constitute the group with the third highest lipogenic activity. Lectins from Bauhinia purpureas, horse gram potato and lima bean had slight lipogenic activity while the remaining lectins had minimal lipogenic activity (Table 3-13).

When these lectins with lipogenic activity but without antilipolytic activity in rat adipocytes were tested in hamster adipocytes for antilipolytic activity, it was found that

Table 3-10

Molecular weights and carbohydrate specificities of the lectins studied

Lectin	Molecular Weight	Carbohydrate Specificity
Concanavalin A	102,000	glucose, mannose
Pisum sativum (garden pea)	I:4,000; II:53,000	glucose, mannose
Lens culinaris (lentil)	52,000	glucose, mannose
Triticum vulgaris (wheat germ)	36,000	N-acetylglucosamine
Solanum tuberosum (potato)	120,000	N-acetylglucosamine
Tetragonolobus purpureas (asparagus pea)	A: 120,000 B: 390,000	fucose
Ulex europeus (gorse)	110,000	fucose
Arachis hypogaea (peanut)	110,000	galactose
Cytisus sessilifolius	247,000	galactose
Bandeiraea simplicifolia	114,000	galactose
Bandeiraea simplicifolia II	114,000	galactose
Momordica charantia (bitter gourd)	129,000	galactose
Bauhinia purpureas	195,000	N-acetylgalactosamine
Wisteria floribunda	136,000	N-acetylgalactosamine
Sophora japonica	138,000	N-acetylgalactosamine
Dolichos biflorus (horse gram)	124,000	N-acetylgalactosamine
Glycine max (soybean)	120,000	N-acetylgalactosamine
Phaseolus limensis (lima bean)	241,000	N-acetylgalactosamine
Maclura pomifera (osage orange)	124,000	N-acetylgalactosamine

Table 3-11

Comparison of antilipolytic activities of various lectins in rat adipocytes

Lectin [a]	% of epinephrine (0.33 μg) -stimulated lipolysis [b]
Momordica charantia lectin	40.47 ± 0.35
Concanavalin A	60.37 ± 1.2
Garden pea lectin	58.41 ± 0.28
Lentil lectin	56.38 ± 0.43
Wheat germ lectin	70.44 ± 1.42
Wisteria floribunda lectin	68.99 ± 0.84

a: All lectins were tested at a dose of 1 nanomole.

b: Epinephrine bitartrate (0.33 μg) elicited an increase of glycerol production over the control at the rate of 4.29 ± 0.11 μmole glycerol per hour per gram fat cell dry weight.

Table 3-12

Effects of various lectins on lipolysis in rat adipocytes

Lectin	% of epinephrine bitartrate (0.33 μg)-induced lipolysis[a]		
	100 μg	20 μg	4 μg
Concanavalin A	56.96 ± 0.33	51.19 ± 0.50	74.31 ± 1.09
Asparagus pea lectin	97.59 ± 1.69	98.32 ± 1.61	96.74 ± 1.55
Bandeiraea simplicifolia II lectin	107.54 ± 3.41	113.36 ± 2.87	105.03 ± 2.41
Cytisus sessilifolius lectin	107.81 ± 1.25	106.70 ± 1.67	107.44 ± 1.43
Bauhinia purpureas lectin	101.52 ± 1.46	102.62 ± 2.13	103.82 ± 0.65
Lima bean lectin	106.34 ± 1.26	104.45 ± 3.46	111.06 ± 5.9
Osage orange lectin	111.92 ± 5.11	102.41 ± 2.80	109.22 ± 1.69

a: Epinephrine bitartrate (0.33 μg) elicited an increase of glycerol production over the control at the rate of 10.17 ± 0.18 mole glycerol per hour per gram fat cell dry weight.

Table 3-13

Comparison of lipogenic activities of various lectins

Lectin [a]	% of added counts per 10 mg lipid [b]
Concanavalin A	27.46 ± 0.73
Lentil	23.52 ± 0.49
Garden pea	22.02 ± 0.08
Wheat germ	18.25 ± 0.22
Momordica charantia	13.73 ± 0.51
Osage orange	12.01 ± 0.34
Wisteria floribunda	9.78 ± 0.70
Bauhinia purpureas	2.99 ± 0.18
Horse gram	2.47 ± 0.10
Potato	1.97 ± 0.13
Lima bean	1.62 ± 0.20
Sophora japonica	1.19 ± 0.45
Bandeiruea simplicifolia	1.12 ± 0.13
Peanut	1.07 ± 0.07
Cystisus sessilifolius	0.87 ± 0.17
Soybean	0.74 ± 0.01
Bandeiraea simplicifolia II	0.61 ± 0.06
Asparagus pea	0.42 ± 0.13
Gorse	0.26 ± 0.13

a: All lectins were tested at a dose of 1 nanomole.

b: D-[3-3H]-glucose (94038 ± 384 cpm) and around 1.2×10^5 fat cells (5.44 mg lipid) were added to each tube.

B. purpureas and horse gram lectins were active but that the osage orange lectin and the gorse lectin remained inactive (Table 3-14).

The antilipolytic effects of both Con A and wheat germ agglutinin could be overcome by increasing the dose of epinephrine, the lipolytic agent (Figure 3-3, 3-4). This suggests that both lectins bind competitively to epinephrine receptors. The ability of M. charantia lectin to competitvely inhibit the lipolytic action of epinephrine has already been demonstrated in Chapter two. Since it has been demonstrated (Cuatrecasas and Tell, 1973) that the antilipolytic actions of Con A, wheat germ agglutinin and M. charantia lectin could be reversed by α-methyl-D-glucoside, N-acetylglucosamine and galactose respectively, it can be inferred that the β-adrenergic receptor on rat epididymal adipocytes is a glycoprotein containing mannose, N-acetylglucosamine and galactose residues.

On the other hand, raising the concentration of corticotropin was ineffective in reversing the inhibitory actions of the two lectins i.e. the lectins are noncompetitive inhibitors of the lipolytic action of corticotropin (Figure 3-5 & 3-6). These observation together with the earlier finding that M. charantia lectin noncompetitively inhibits the lipolytic action of corticotropin suggest that galactose, mannose and N-acetylglucosamine residues may be absent from the corticotropin receptor on rat adipocyte.

The three mannose binding lectins, Con A, garden pea lectin, and lentil lectin, were approximately equipotent in their antilipolytic and lipogenic activities. This group of lectins

Table 3-14

Effects of various lectins on lipolysis in hamster adipocytes

	Dose (μg)	% of 0.5 mU ACTH-induced lipolysis
Concanavalin A	300 μg	0
	100 μg	57.06 ± 3.04
Momordica charantia lectin	300 μg	0
Wisteria floribunda lectin	300 μg	9.56 ± 1.52
Bauhinia purpurea lectin	300 μg	87.64 ± 7.69
Horse gram lectin	300 μg	47.80 ± 3.16
Osage orange lectin	300 μg	101.42 ± 2.35
gorse lectin	110 μg	142.24 ±15.92
propranolol	100 μg	34.60 ± 9.87
insulin	1 mU	79.37 ± 3.95

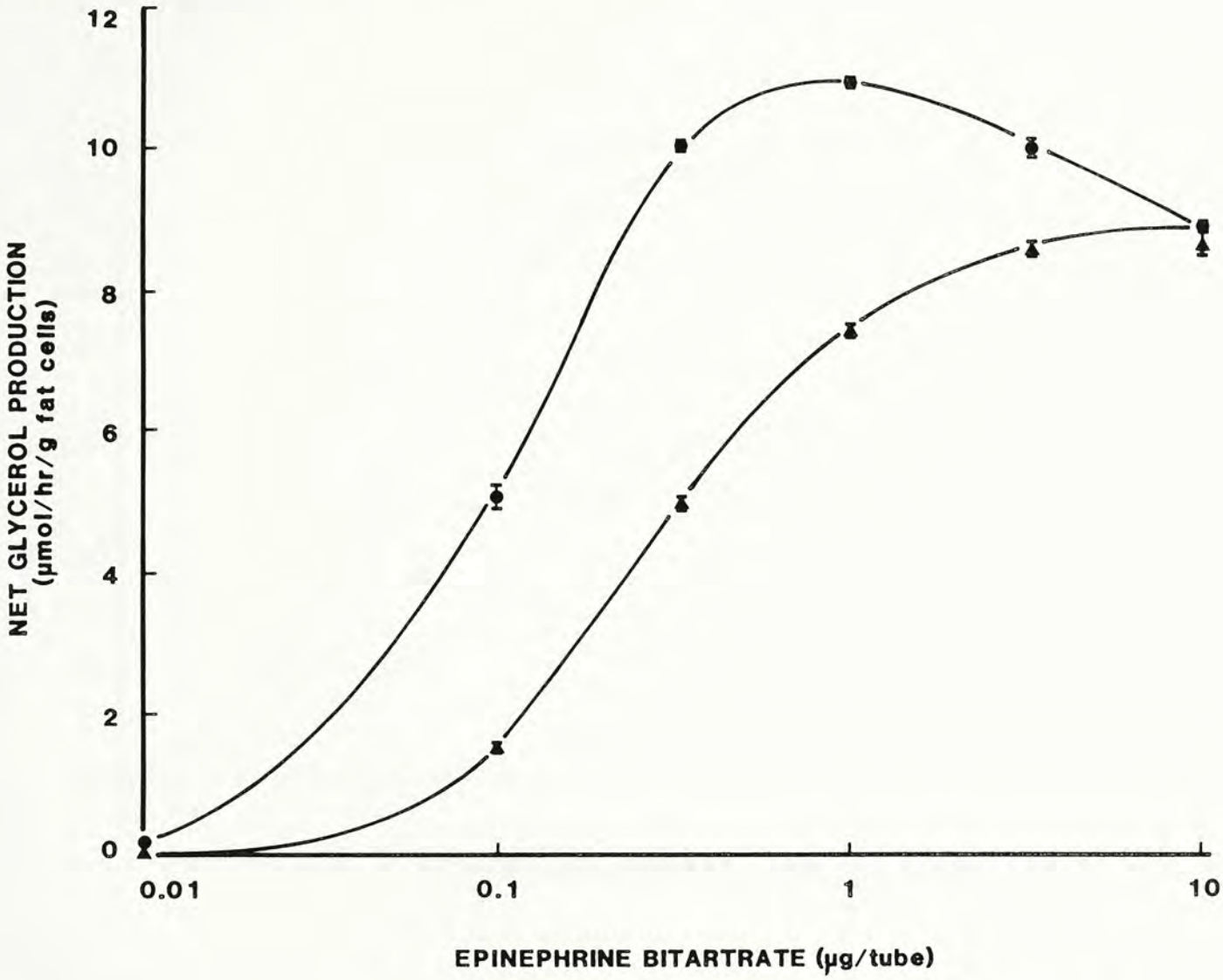


Figure 3-3. Effect of increasing doses of epinephrine bitartrate (●) on the antilipolytic effect of 50 µg Con A (▲). The inhibitory effect of Con A diminished at high doses (3 and 5 µg) of epinephrine and finally was overcome by a dose of 10 µg.

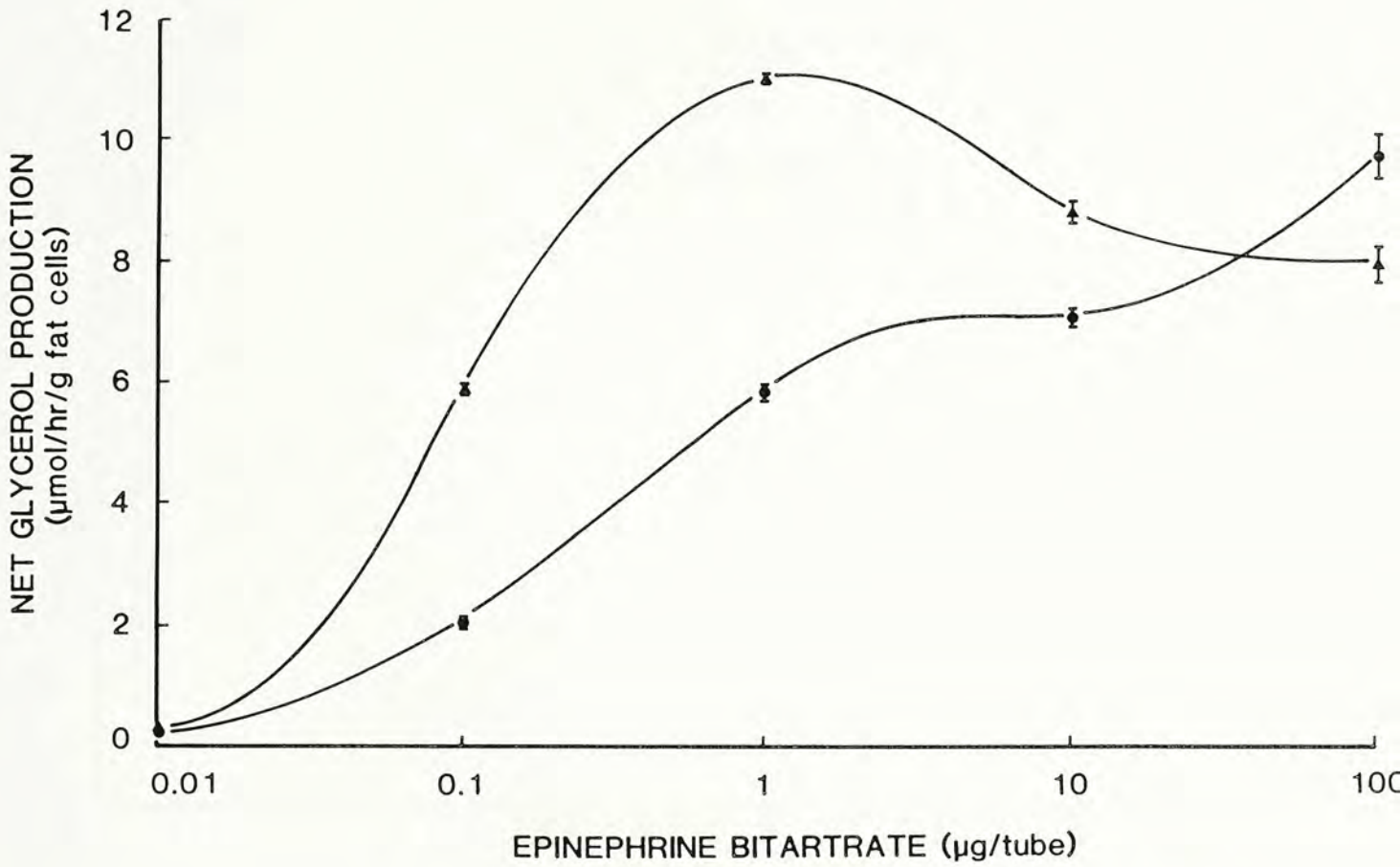


Figure 3-4. Effect of increasing dose of epinephrine bitartrate (▲) on the antilipolytic effect of 0.25 nmole (9 µg) wheat germ agglutinin (●) . The inhibitory effect of wheat germ agglutinin was overcome at high dose of the hormone.

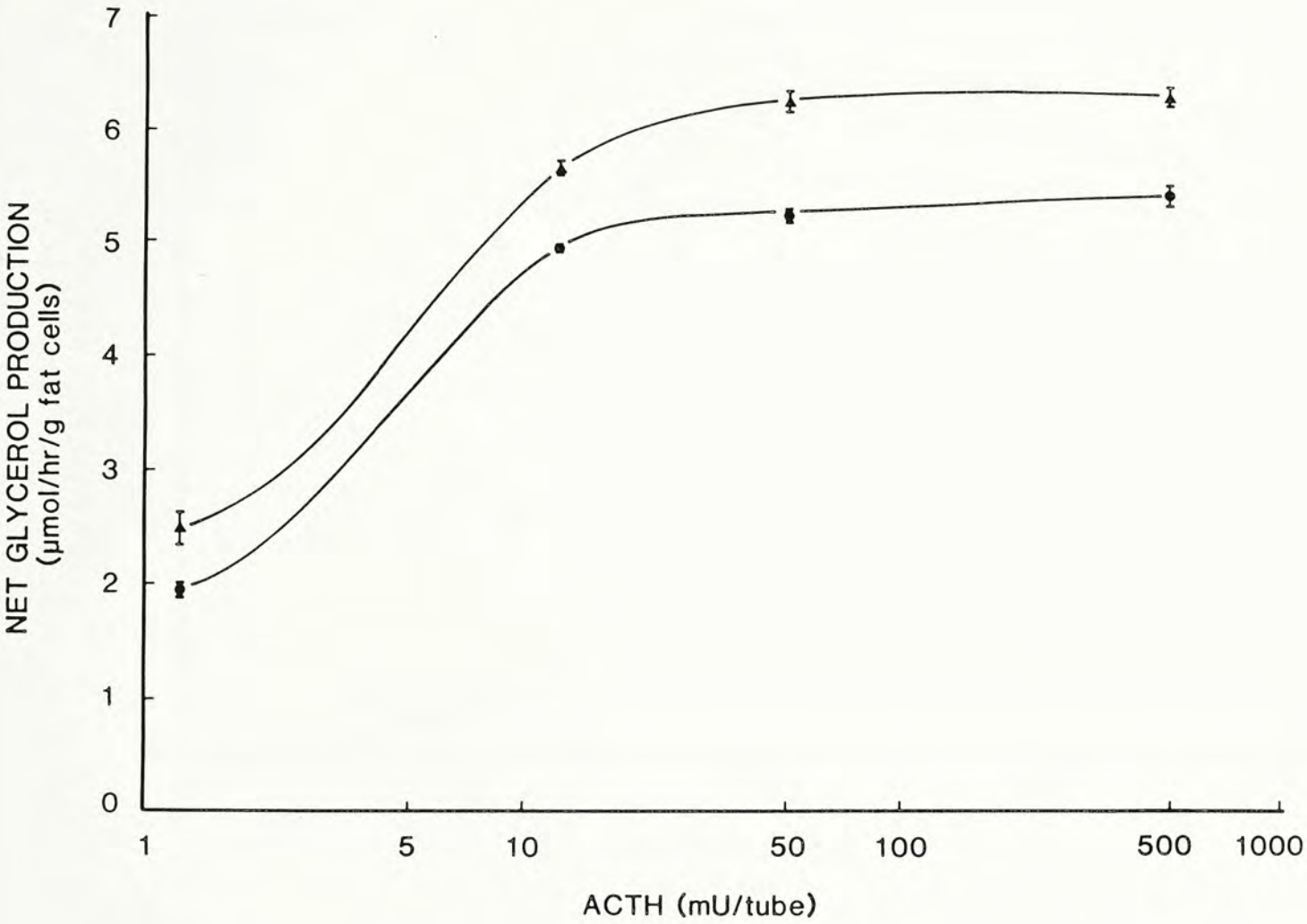


Figure 3-5. Effect of increasing doses of ACTH (▲) on antilipolytic effect of 100 µg Con A (●). The inhibitory effect of Con A could not be overcome by increasing the dose of the hormone.

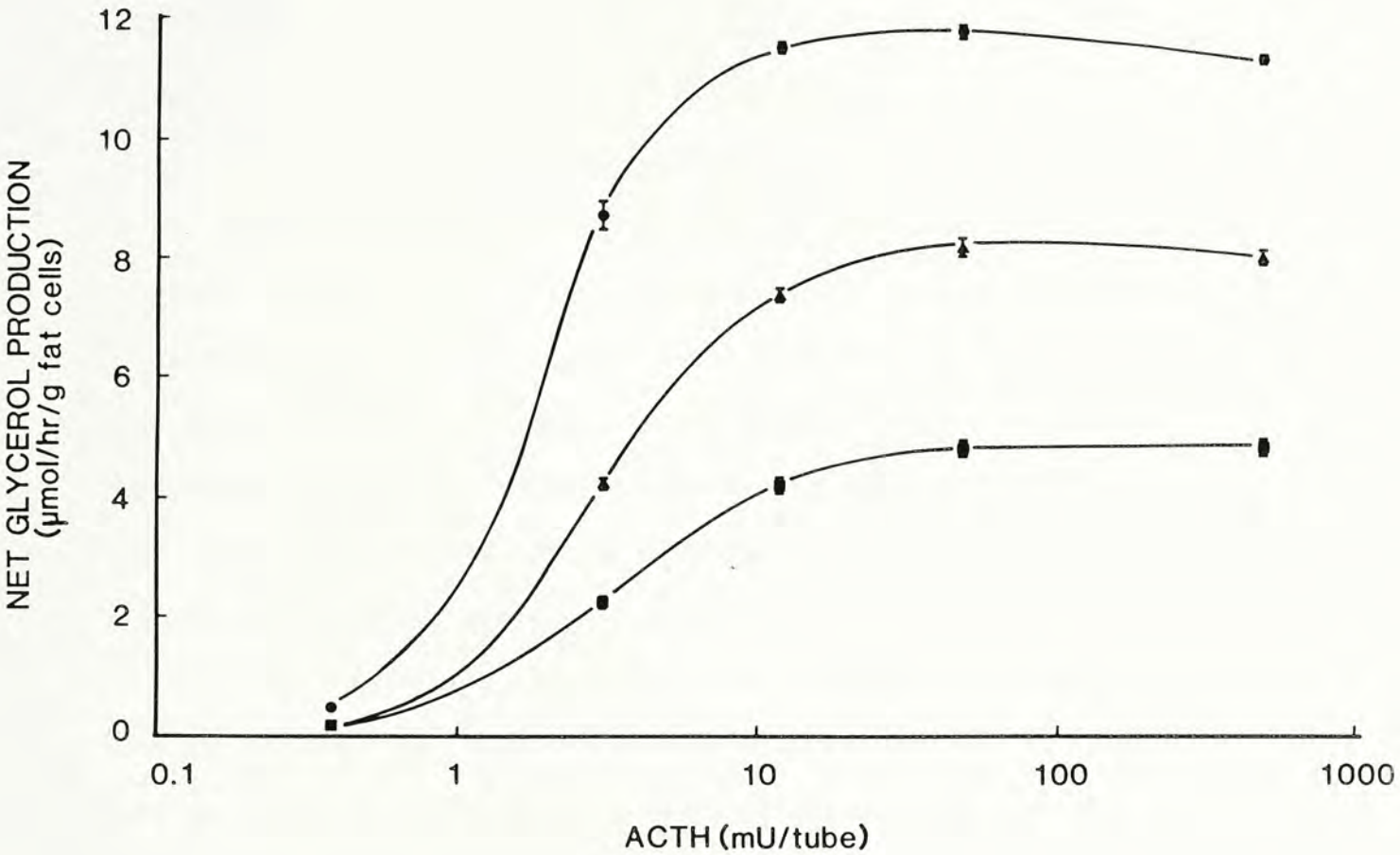


Figure 3-6. Effect of increasing doses of ACTH (●) on antilipolytic effect of 0.25 nmole wheat germ agglutinin (■). The inhibitory effect of wheat germ agglutinin could not be overcome by increasing the dose of the hormone. It is noteworthy that the antilipolytic effect of wheat germ agglutinin was more potent than that of M. charantia lectin (L_5) (▲) when the same dose (0.25 nmole) of L_5 was used.

had the highest lipogenic activity and the second highest antilipolytic activity of all the lectins examined.

The N-acetylgalactosamine binding wheat germ lectin markedly enhanced lipogenesis from glucose in rat adipocytes, in agreement with the observation of Katzen et al. (1981) that the lectin stimulated glucose oxidation. The antilipolytic effect of wheat germ lectin reported by Katzen et al (1981) was confirmed in this study. The lower lipogenic activity of the N-acetylglucosamine binding potato lectin was probably related to its lower degree of purity. Nevertheless, the insulin-like activities of the two N-acetylglucosamine binding lectins agree well with the reported ability of wheat germ lectin to bind to insulin receptors (Katzen et al., 1981; Hedo et al., 1981).

By examining galactose and N-acetylgalactosamine binding lectins that have not been previously studied, we were able to procure evidence confirming the findings of Katzen et al. (1981) that most lectins in this category were devoid of any significant antilipolytic effect in rat adipocyte. The lectins that we so tested included the galactose binding Bandeiraea simplicifolia II and Cytisus sessilifolius lectins and the N-acetylgalactosamine binding lectins from Bauhinia purpurea, Phaseolus limensis and Maclura pomifera. Minimal lipogenic activity was elicited by the galactose binding lectins from B. simplicifolia, peanut and Cytisus sessilifolius and the N-acetylgalactosamine lectins from soybean, Sophora japonica and P. limensis. These findings were consistent with the observation of Katzen et al. (1981) that B. simplicifolia, peanut soybean and

Sophora japonica lectins did not stimulate glucose oxidation. The N-acetylgalactosamine enhanced lipogenesis slightly while Wisteria floribunda and Maclura pomifera lectins were strongly lipogenic. It is noteworthy that M. pomifera lectin possessed potent lipogenic activity and yet lacked significant antilipolytic activity in the rat. This dissociation between antilipolytic and lipogenic activities probably comes from the different mechanisms required to produce the two effects. The regulation of the intracellular level of cyclic AMP, the messenger for the lipolytic hormones, determines the rate of lipolysis while promotion of glucose uptake and activation of lipogenic enzymes ensues in enhanced lipogenesis.

There was an interesting finding when the antilipolytic activities of some of the lectins in the hamster adipocyte system were investigated. Although Concanavalin A, M. charantia lectin and Wisteria floribunda lectin were active while M. pomifera and gorse lectins were inactive in both systems, B. purpurea and horse gram lectins which were devoid of antilipolytic activity in the rat system possessed activity in the hamster system. The disparity in the findings on adipocytes from the two rodent species suggests that B. purpurea and horse gram lectins do not act as β-adrenergic antagonists but may inhibit lipolysis in hamster adipocytes by interaction with the corticotropin receptor or the α-adrenergic receptor. Lectins inactive in both systems apparently do not interact with receptors to the lipolytic hormones.

Outstanding among the galactose binding lectins is the M. charantia lectin. Of all the lectins which exert

antilipolytic activity in rat adipocytes in this study, the M. charantia lectin had the highest antilipolytic potency although it ranked third as far as its lipogenic potency is concerned. The mannose binding lectins belong to the group with the second highest antilipolytic activity. The third group comprising the N-acetylgalactosamine binding W. floribunda lectin had slightly lower antilipolytic activity than the second group. The antilipolytic and lipogenic activities of M. charnatia and W. floribunda lectins in the rat are reminiscent of a report of insulin-like activity of ricins I and II by Hedo et al. (1981). This group of investigators reasoned that the ability of ricins I and II to trigger responses typical of those to insulin is dependent on the β conformation of the galactose residue. It is possible that the M. charantia and W. floribunda lectins possess the requisite β-galactose residue.

We examined two fucose binding lectins, one from gorse and other from asparagus pea. In keeping with the observations of Katzen et al. (1981) on the gorse seed lectin and asparagus pea lectin lacked antilipolytic activity in rat adipocytes. Similar results were obtained with gorse lectin when hamster adipocytes were utilized instead. These results indicate that the fucose binding lectins did not antagonize binding of lipolytic hormones to their receptors in either rat or hamster adipocytes. The two lectins also produced minimal stimulation of lipogenesis. Katzen et al. (1981) have noted that the gorse lectin fails to bind to insulin receptors or stimulate glucose oxidation in rat adipocytes.

To summarize, results of the present study largely confirm and extend those of Katzen et al. (1981). We have found that mannose binding and N-acetylglucosamine binding lectins exhibit potent insulin-like activity. Certain galactose and N-acetylgalactosamine binding lectins also manifest insulinomimetic activities but the majority of lectins in this category are inactive probably because their galactose residue lack the requisite conformation. The fucose binding lectins are devoid of any antilipolytic or lipogenic activity. The insulin-like activities of these lectins have previously been demonstrated to be abolished by the simultaneous addition of the specific carbohydrates, indicating that the activities are mediated by interaction with the carbohydrate components of membrane receptors (Hedo et al., 1981). The correlation between the insulin-like bioactivities and insulin receptor binding abilities has further led to the observation that the insulin receptor contains mannose, galactose, N-acetylglucosamine and N-acetylgalactosamine in its carbohydrate moiety (Hedo et al., 1981).

4. OVERALL DISCUSSION

4 OVERALL DISCUSSION

The effects of a saponin, a lectin and two abortifacient proteins all firstly isolated from M. charantia seeds in our laboratory on lipid metabolism in isolated rat adipocytes have been studied. Compounds of similar chemical nature from T. kirilowii (Tianhuafen), ginseng and other plants have also been examined to see how their effects on lipid metabolism compared with those of M. charantia seed compounds.

4.1 Abortifacient proteins

The apparent suppressive effect of α- and β-momorcharins on corticotropin-induced lipolysis can be ascribed to the inactivation of the hormone rather than an interaction with the hormone receptors on the adipocytes.

Tricosanthin resembles α- and β-momorcharins in that it does not elicit any lipolytic, lipogenic, antilipolytic or antilipogenic activities. Because there is a close correlation between the in vitro and in vivo metabolic effects of hormones like epinephrine, insulin and corticotropin-related peptides (Rodbell 1964; Moody et al., 1974; Ng et al., 1982; Natelson et al., 1963), it is highly likely that these abortifacient proteins of plant origin lack the ability to influence lipid mobilization or biosynthesis when administered in vivo. It is perhaps noteworthy that these proteins elicit abortifacient activity when administered to pregnant mice at a dosage of 100 µg or less per animal and produce adverse effects in embryonic development in vitro when present at a concentration of 30 µg/ml or less

(Chan, 1984). Thus it would be fair to conclude that at doses higher or comparable to those effectively used in inducing abortion, the proteins did not evoke observable changes in lipolysis and lipogenesis.

4.2 Saponins

Saponins are a class of steryl glycosides found in higher plants. They are poisonous to lower forms of life but practically nontoxic to man if ingested (McIlroy, 1951). The effects of M. charantia seed saponin and three ginseng saponins on lipid metabolism have been investigated. The M. charantia seed saponin acts as a noncompetitive inhibitor of corticotropin, glucagon and epinephrine in lipolysis. Incorporation of tritiated glucose into total lipids was inhibited. Adipocyte viability was not affected by the saponin suggesting that its inhibitory effect on lipolysis and lipogenesis was not due to a decrease in cell viability. Ginsenoside Rg_1, Rb_2 and Rc inhibited corticotropin-induced lipolysis in a noncompetitive manner. They also suppressed dbcAMP-induced lipolysis similar to the seed saponin. But the basal glucose incorporation into lipid was not affected. Thus, the site(s) of the antilipogenic effect of seed saponin was probably due to an inhibition of the glucose transport into the adipocytes while that of the ginsenoside Rg_1 and Rc was due to inhibition of the insulin-stimulated glucose transporter. Although saponin(s) were also detected in fruits (data from our laboratory), the possibility that the saponin(s) play an active role in the hypoglycemic activity of M. charantia fruit is doubtful. Its antilipolytic effect probably due to

inhibition of glucose transport may prevent peripheral glucose uptake into adipose tissue and thus worsen the hyperglycemia symptom of diabetes. This is contrary to the finding that M. charantia fruit extract lowers blood glucose by facilitating periperal carbohydrate utilization. Since ginsenoside Rg_1, Rb_2 and Rc did not affect lipid metabolism, they alone cannot account for the hypoglycemic activity of ginseng.

4.3 Lectins

The effects of lectins that specifically bind mannose, fucose, galactose, N-acetylgalactosamine and N-acetylglucosamine respectively, on lipolysis in isolated hamster and rat adipocytes and lipogenesis in rat adipocytes, were examined. The relative potencies of the lectins in antagonizing epinephrine-induced lipolysis in rat adipocytes were M. charantia lectin (galactose binding) > Concanavalin A, garden pea lectin, lentil lectin (mannose binding) > wheat germ lectin (N-acetylglucosamine binding) and Wisteria floribunda lectin (N-acetylgalactosamine binding). The relative lipogenic potencies of the lectins were Concanavalin A, garden pea lectin, lentil lectin > wheat germ lectin > Maclura pomifera lectin (N-acetylgalactosamine binding), Wisteria floribunda lectin, and Momordica charantia lectin. The fucose binding gorse and asparagus pea lectins had minimal antilipolytic and lipogenic activities. The galactose binding horse gram and Bauhinia purpurea lectins had slight lipogenic activity and no antilipolytic activity in rat adipocytes but they exerted antilipolytic activity in hamster adipocytes. Other

galactose and N-acetylgalactosamine binding lectins tested including Cytisus sessilifolius, peanut Bandeiraea simplicifolia II, soybean, Phaseolus limensis and Sophora japonica exhibited minimal antilipolytic and lipogenic activities.

Except M. charantia, whose fruit also contains lectin (data from our laboratory), all the plants from which the stated lectins with insulin-like activities are isolated have not been used as folkloric diabetic remedies. Folkloric hypoglycemic plants are usually used in infusion or decoction form in the treatment of diabetes. Since lectins are usually present in plants in very low amount and they are labile to enzymes and heat, lectins present in M. charantia and Tianhuafen may not be responsible for the hypoglycemic effect of the infusion or decoction though they exhibit potent in vitro insulinomimetic effect.

4.4 Insulin-like peptides

As mentioned before, Khanna et al. (1981) claimed that they had isolated a potent hypoglycemic peptide called "polypeptide-p (p-peptide)" from fruits, seeds and tissue culture by using the same purification protocol. We strictly followed the protocol to isolate this "p-peptide" from fruits and seeds and found that the fruit "p-peptide" preparation was fairly homogeneous and exhibited lipogenic effect. It had potent antilipolytic effect in hamster adipocytes but not in rat adipocytes. The seed "p-peptide" preparation was heterogeneous and contained saponin which accounted for the potent antilipolytic and antilipogenic effect of seed "p-peptide". Following further fractionation of seed "p-peptide" by affinity

chromatography on fetuin agarose, a fraction with lipogenic effect could be prepared.

By using the insect insulin purification scheme, four fractions (IC-3, IC-4, IC-5 and IC-7) with lipogenic effect were isolated from the seed. They exhibited antilipolytic effect in hamster adipocytes but not in rat adipolytes. Owing to the fact that these preparations contained may nonproteinaceous compounds detected by agarose electrophoresis, further purification work did not continue.

By using the modified mammalian insulin like purification protocol, two relatively pure proteins designated C_{1-3} and C_{6-1} were isolated. C_{1-3} was acidic and behaved similar to insulin while C_{6-1} was basic and moved in a direction opposite to insulin in agarose electrophoresis. Both proteins exhibited potent lipogenic and antilipolytic activity in rat adipocytes. However, subcutaneous administration of 2 mg/Kg dose of C_{1-3} and 5 mg/Kg dose of C_{6-1} to aged rats did not cause any significant lowering of plasma glucose and total lipid levels.

The observation of antilipolytic and lipogenic activities in fruit "p-peptide" and lipogenic activity in a fraction derived from seed "p-peptide" and the isolation of one basic and one acidic lipogenic proteins indicate the presence of, in *M. charantia* fruits and seeds, non-lectin compounds that could be extracted by acid-ethanol and exhibited insulinomimetic activity *in vitro*. Whether the insulin like protein (C_{1-3}) isolated from seeds is the same as those found in the fruit as reported by the groups in Mainland China still remains to be

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investigated because purification protocol of these peptides has not yet been published (Zhang, personal communication).

It would be premature to conclude that the two proteins isolated from M. charantia seeds possessing lipogenic and antilipolytic properties are the active components responsible for the hypoglycemic effect of M. charantia fruit. More experiments should be carried out in the future to investigate whether they are also present in the fruit and their in vitro and in vivo biological activities. These include glucose uptake, glycogen synthesis, insulin receptor binding, insulin radioimmunoassay and in vivo experiments to show that they are really hypoglycemic in diabetic animals and men.

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